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THE TIME FACTOR IN FERTILIZATION AND EMBRYO DEVELOPMENT IN THE SUGAR BEET [1]

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INTRODUCTION

Although investigations during the past decade have conclusively shown that the embryo sac of the sugar beet (*Beta vulgaris* L.) is mature at the time of anthesis and that the pollen is viable and the stigma receptive even before the anthers dehisce, the time factor in fertilization and embryo development has remained a debatable issue; therefore, it was the purpose in this investigation to study, on a large amount of material, the sequence of the nuclear phenomena in the embryo sac from the time of anthesis to the development of the embryo.

MATERIAL AND METHODS

The material for study was obtained from a 10-acre field of mother beets of the Flat Foliage variety grown on the farm of the American Beet Sugar Co. at Rocky Ford, Colo. Practically all the flowers were collected during the height of the blooming period between the 8th and the 20th of June in 1931 and 1932. Several hundred plants were selected and marked by numbered stakes. On each of these plants from 5 to 15 flowers were marked. The location of these flowers was indicated by a small numbered tag placed around the branch of the floral axis near the flower cluster in question. The flower cluster itself was marked by placing an india-ink dot on the subtending bract. In every case the oldest flower of a cluster was chosen principally to avoid mistakes in collecting. Flowers were usually marked in the morning, since at that time anthesis is most general, and collections were made at intervals of several hours, the latest collection being made 14 days after anthesis. No later collections were found necessary, since in material 12 to 14 days old the embryos are mature.

The material marked for study was separated with a sharp safety-razor blade from the other members of a flower cluster and dropped immediately into a vial with fixing solution. Most flowers were killed in Carnoy's solution, but a few hundred of the 1932 collection were fixed in a modification of Nawaschin's solution to bring out more clearly the location of the pollen tubes. The larger amount of the material was embedded in paraffin after being taken through the xylol-alcohol series. The results obtained were not inferior to those with the butyl alcohol technic, although the latter reagent somewhat facilitated the cutting of flowers with old embryos.

All cytological material was stained with Heidenhain's iron-alum haematoxylin. The photomicrographs were taken on Wratten and Wainwright M plates with B-58 and E-22 filters used singly or in combination. The drawings [2] are based on photomicrographs.

[1] Received for publication July 7, 1933; issued February, 1934.

[2] Credit is due Mrs. E. Artschwager for the preparation of all drawings in this paper.

MORPHOLOGY OF THE BEET FLOWER

The small sessile flowers of the sugar beet are usually borne in clusters of two or more which are attached to the inflorescence axis or secondary branches of the latter. The flowers are perfect; the calyx is 5-parted and incurved, adhering to the base of the ovary and becoming hard in the fruit. There are five stamens. The 3-carpellary ovary is sunk in a fleshy disk enclosing a campylotropous ovule which is attached laterally to the ovary wall by a short funiculus. The style is very short, terminating in a 3-, sometimes 4- or 5-lobed stigma which persists in the fruit.

While most beet flowers, under Colorado conditions, open soon after 7 a.m., anthesis continues throughout the day. Similar observations are recorded by Oksijuk (*6*) [3] for the beet-growing regions of Russia. As the flowers open, the sepals either expand completely to form an angle of 90° with the floral axis or they open up only partially. In either case the sepals close again more or less completely (fig. 1, *E*, *F*) after embryo development is initiated. As the sepals begin to expand the anthers open by longitudinal fissures. The fresh pollen grains at first adhere together in clumps, but these soon dry and the pollen is carried by wind and insects to the receptive stigmas of other flowers. A blooming beet field is alive with visiting insects, and since in the morning hours the atmosphere is usually calm, air currents may often be only of minor importance in the transfer of pollen from flower to flower and from plant to plant.

OPENING OF STIGMA LOBES

At the time of anthesis the stigma has the form of an erect conical tube (fig. 1, *A*) composed of a number of folded lobes. In the majority of flowers this number is 3; not infrequently, however, there are 4 (fig. 1, *B*, *C*, *D*) and even 5. In such cases the expanded stigma is not symmetrical, as some of the lobes are larger than others. Van Heel (*4*) also reported the occurrence of multilobed stigmas in some of his material. The stigma lobes begin to unfold some time after the flower has opened; this process is a gradual one, and the time necessary for complete expansion varies with individual flowers. According to early writers on sugar-beet breeding, the stigma begins to unfold the day after flowering and the stigma lobes are fully expanded only after the third day. Recent observations reported by Oksijuk (*6*) place the first indication of unfolding at 5½ hours after anthesis, with the entire process completed in 24 hours.

Some data on the opening time of the stigma lobes under Colorado conditions are reported in table 1. Of 54 flowers examined 7 hours after anthesis, about one fourth had stigma lobes still closed. Another lot of 61 flowers, examined after 24 hours, showed most stigmas at various stages of unfolding, with only a small percentage remaining unchanged. A final observation made 36 hours after anthesis showed practically all flowers with stigma lobes fully expanded. Earlier results obtained in 1931 (but not recorded in table 1) indicate that the stigma lobes of some flowers started to open as early as 5 hours after anthesis and had completely expanded several hours later. In general, however, the stigma begins to unfold in midafternoon, or about 7 hours after the flowers open, and expansion is completed

[3] Reference is made by number (italic) to Literature Cited, p. 843.

within 36 hours. Just as the sepals of a beet flower occasionally fail to fold back completely, so the stigma lobes at times only partially

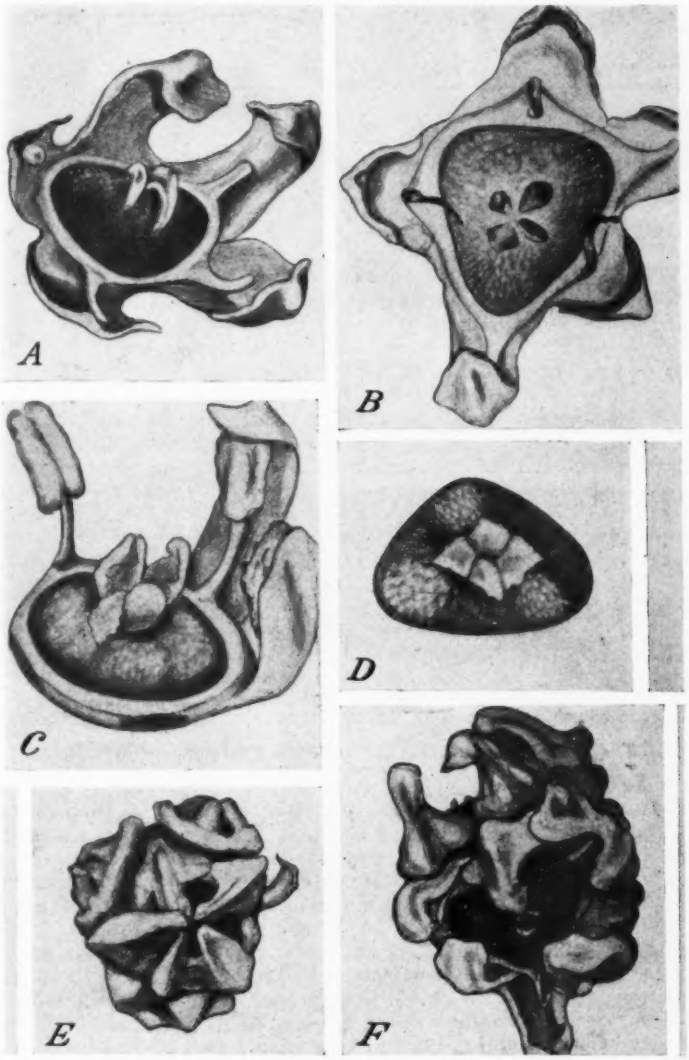


FIGURE 1.—*A*, Young beet flower with anthers removed, seen from above, the stigma lobes just beginning to expand; *B*, 3-day-old flower with stigma lobes fully expanded; *C*, 1-day-old flower with stigma lobes partly expanded; *D*, 2-day-old flower with stigma lobes fully expanded; *E*, partly mature seed ball with sepals closed tightly; *F*, partly mature seed ball with sepals folded back only partly.

expand. This latter condition is not infrequent and may be the result of early growth of the pollen tubes, although observations supporting such a correlation have not as yet been made.

TABLE 1.—*Opening of the stigma lobes*

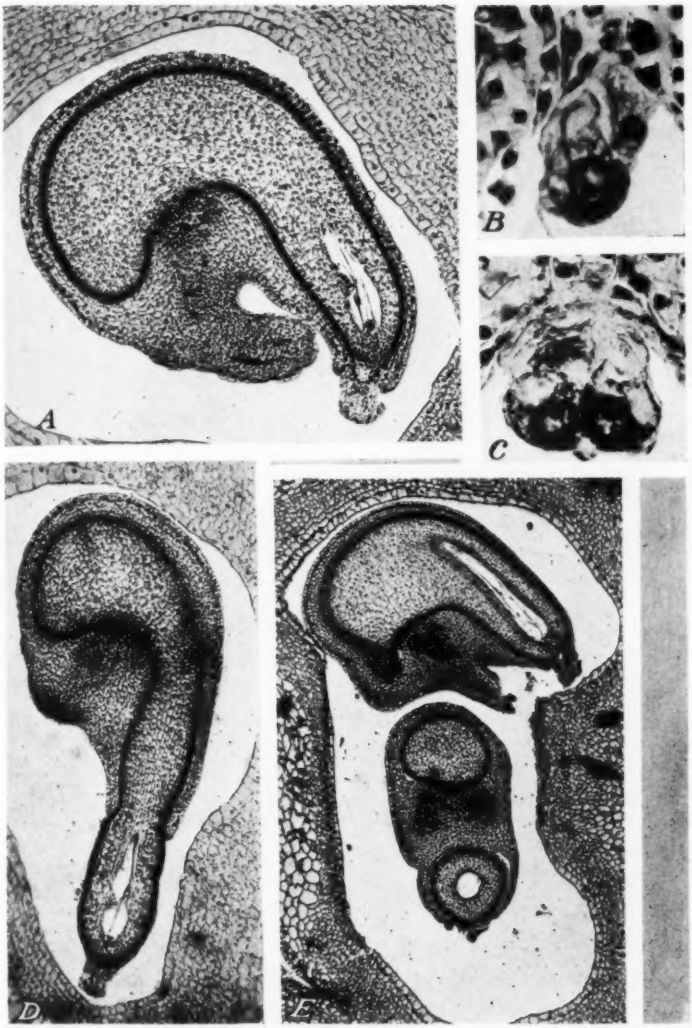
Time after anthesis	Plant no.	Flowers examined	Flowers with stigma lobes— Closed	One fourth open	One half open	Open
7 hours	1	4		4		
	2	10		10		
	3	5		5		
	4	4		4		
	5	3		3		
	6	2		2		
	7	5	3	2		
	8	5	3	2		
	9	5	5			
	10	3			3	
	11	3		1	2	
	12	3	3			
	13	2		2		
Total		54	14	35	5	
Percent			25.9	64.8	9.3	
24 hours	14	4			1	3
	15	5			5	
	16	5			5	
	17	4			4	
	18	3			3	
	19	5	2	3		
	20	5	1	4		
	21	5		4	1	
	22	4				4
	23	3			2	1
	24	2			2	
	25	2	1	1		
	26	2		2		
	27	5		5		
	28	2		1	1	
	29	2		1	1	
	30	3			2	1
Total		61	4	21	27	9
Percent			6.5	34.4	44.3	14.8
31 hours	31	4	3	1		
	32	5		2	3	
36 hours	33	5			3	2
	34	5				5
	35	5				5

ORGANIZATION OF EMBRYO SAC AT TIME OF FLOWERING

The solitary ovule of the beet flower is campylotropous and lies horizontally in the cavity of the ovary, with its largest diameter at the time of anthesis a little over 0.5 mm. The micropylar tip of the ovule is usually bent slightly upward and often touches the roof of the ovary cavity some distance from the opening of the stylar canal.

The embryo sac at the time of anthesis is a narrow, slightly curved cavity consisting of the embryo sac proper and the caecum (pl. 1, *A*). The latter begins to form before the flower opens, and grows in length at the expense of the nucellar tissue. At the time of anthesis the caecum is as long as the embryo sac proper, but after fertilization it enlarges rapidly, forming a horseshoe-shaped cavity, and attains its final size when the embryo is still very small.

The embryo sac contains the normal nuclear complex. The egg apparatus at the micropylar end of the sac is composed of three more or less pear-shaped cells, the egg and the two synergids (pl. 1, *B*, and fig. 2, *A*). The nucleus of the mature egg usually rests in the basal part of the egg cell and is surrounded by dense cytoplasm. Below the egg nucleus, at the micropylar end, there is commonly present a



A, Horizontal section through ovule at time of anthesis. × 94. *B*, Egg and synergid at time of anthesis. × 560. *C*, Two eggs in embryo sac. × 700. *D*, Ovule with abnormally elongated nucellar tip. × 45. *E*, Two ovules in a single ovary cavity. × 45.

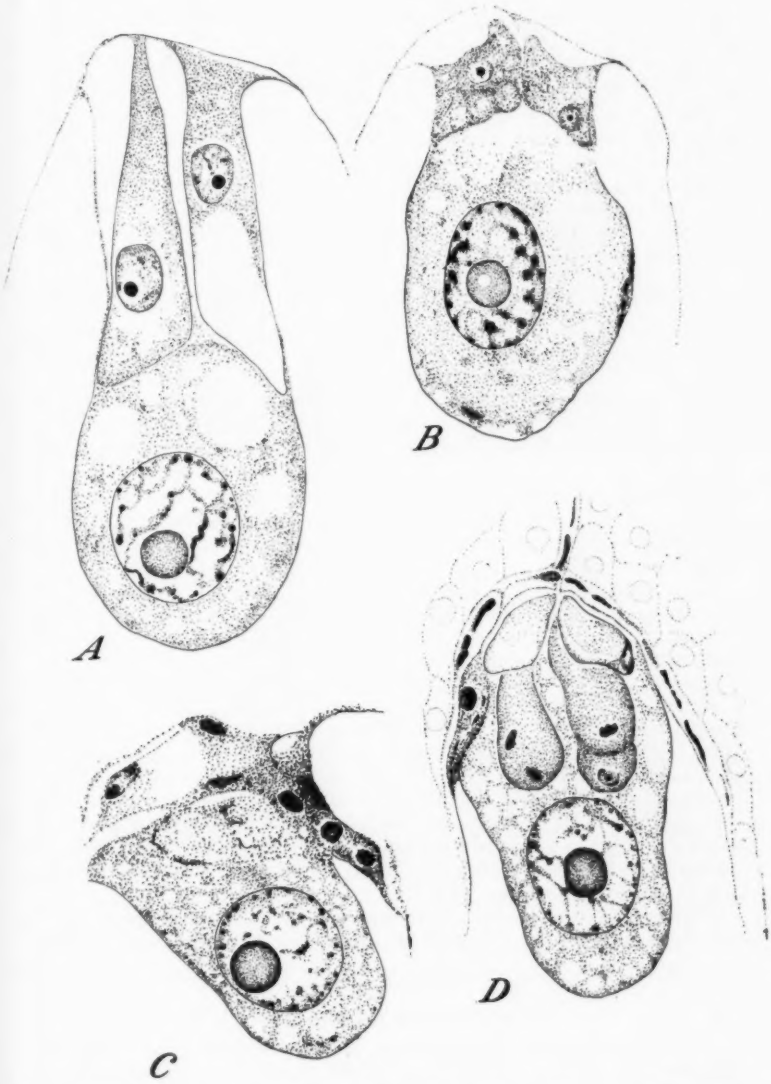


FIGURE 2.—*A*, Egg with synergids at time of anthesis, × 1,440; *B*, egg with synergids degenerating, × 1,440; *C*, fertilized egg with a number of X - bodies, × 1,440; *D*, egg with pollen tubes and degenerated synergids, × 1,440.

large vacuole. The synergids are slightly shorter than the egg, very slender, and contain large vacuoles near the base; their nuclei are small. There are commonly three antipodals at the chalazal end of the sac. This number is often augmented, especially after anthesis, and frequently an antipodal cell may contain two nuclei. Both Favorsky (*3*) and Oksijuk (*6*) observed this multiplication of antipodals, but the latter believed this phenomenon to be uncommon.

The fusion polar or primary endosperm nucleus is very large, and its nucleolus is even more conspicuous than that of the egg. Its position in the embryo sac varies in relation to the antipodals. Of 26 flowers examined at the time of anthesis, 19 showed the fusion polar between the egg and the antipodals, tending rather toward the antipodals; in 2 cases the fusion polar was found opposite the antipodals, and in 5 cases in the caecum below the antipodals.

ABNORMALITIES IN DEVELOPMENT OF OVULE AND EMBRYO SAC

Although the ovary with its ovule and embryo sac develops normally in most flowers, one meets now and then with abnormalities, which, however, are of no practical significance in seed production.

The phenomenon of the development of 2 ovules in 1 ovary cavity (pl. 1, *E*) actually constitutes a coalescence of 2 adjacent flowers, as there is a partial or complete doubling in the number of stamens and stigma lobes, associated, however, with 1 common stylar canal and a single ovary cavity. The two ovules have a normal appearance and may well enough develop into viable seed, although seed balls enclosing two seeds within a common cavity have never been observed.

The occurrence of 2 eggs in an embryo sac (pl. 1, *C*) has been observed only once, and since only 1 synergid accompanied the 2 eggs, it seems quite evident that the supernumerary egg is the sister nucleus of the synergid.

Abnormal elongation of the proximal part of the nucellus (pl. 1, *D*) is sometimes observed. While the inner integument of such ovules accompanies the nucellar protuberance, the outer one ceases growth after having attained its normal development. The embryo sac of such abnormal ovules appears unaffected, and the nuclei are found in their respective positions and, on the whole, show a normal structure.

Suppression of embryo-sac development is not uncommon. The ovules, though normal in appearance, are somewhat dwarfed. The embryo sac is either entirely wanting or is represented by a narrow cavity containing several nuclei.

Favorsky (*3*) reports the occurrence of polyembryony, a phenomenon never observed in the material on which this investigation was based.

POLLEN

SIZE AND SHAPE OF GRAINS

Pollen grains from a given flower, though variable in size, are fairly uniform, as grains of extreme dimensions are numerically few. Oksijuk (*6*) noted in his material two types of pollen, namely, a large percentage of small grains of an average diameter of 16.8μ and a comparatively smaller amount of large pollen with a diameter of 21.5μ. He emphasizes the coexistence of these two types, pointing out that beet races differ not only in their gross morphology but

possibly also in their pollen, and that in seed beets, which commonly represent a mixture of races, the parental pollen types have preserved their identity. The writers' data do not harmonize with those of Oksijuk, since only the plotted measurements of *Beta maritima* × Pioneer hybrid (fig. 3, *B*) show a somewhat bimodal distribution. Different beet races show consistent and even significant differences in the average size of their pollen, and it is not impossible that data on

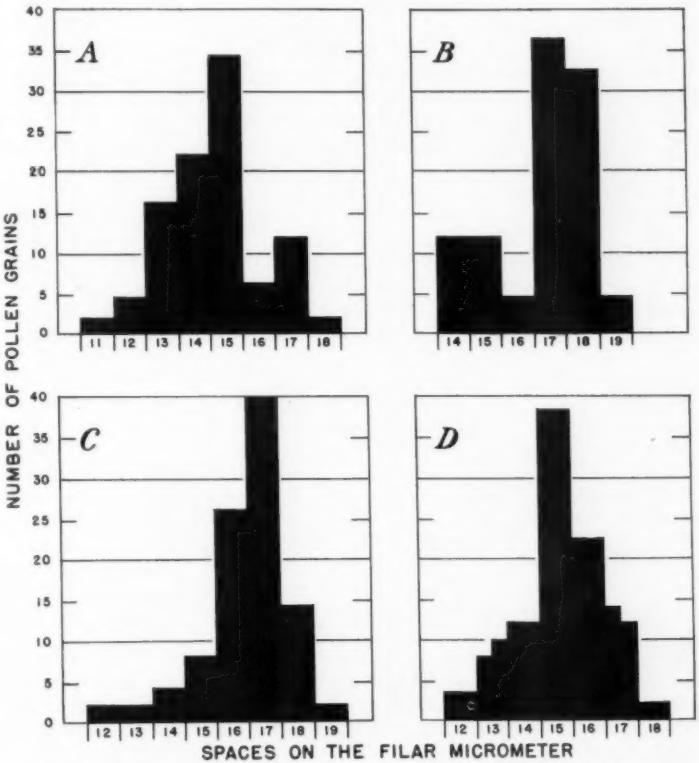


FIGURE 3.—Diameter of 100 pollen grains in each of four sugar-beet selections. Each space on the micrometer is 1.42μ. *A*, Selection no. 10277-O; *B*, selection no. 10274-O; *C*, selection no. 10271-O; *D*, selection no. 10270-O.

size of pollen could be utilized as an auxillary diagnostic character in a taxonomic study of beet races.

The mature pollen grain is spherical, its outer membrane thick and sculptured, with numerous thin spots for the exit of pollen tubes. The microspore nucleus divides several days before the anthers dehisce, and gives rise to the tube nucleus and the two generative nuclei. The tube nucleus is more or less spherical with a small nucleolus and dense cytoplasm; the generative nuclei or sperms (pl. 2, *D*) are slender elliptical bodies with chromatic granules em-

bedded in cytoplasm. Young but mature pollen contains a large number of irregular starch grains, but before anther dehiscence the typical iodine reaction is negative, the starch having been changed to sugar.

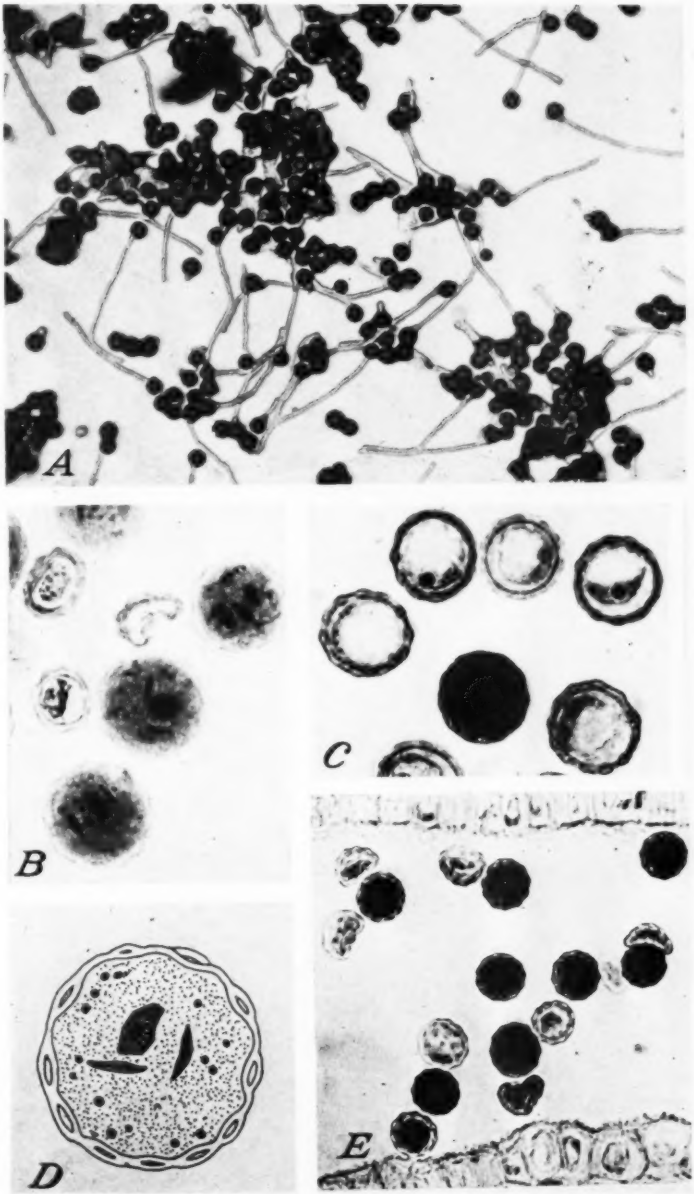
GERMINATION

Since beet pollen was reputed to germinate poorly on artificial media, germination tests were made on the unopened stigma of dehiscing flowers. For this purpose flowers were marked early in the morning, bagged, and examined in groups at 1-hour intervals. Both cotton blue and Nachtblau were found to be selective stains for the pollen tubes of sugar beet, especially if preceded by a short application of acetocarmine. The first germinating pollen grains were observed 2 hours after anther dehiscence. The pollen tubes were short, hardly exceeding the diameter of the pollen grain. Later examinations showed pollen tubes of increasing length with their tips already buried between the papillae of the stigma lobes.

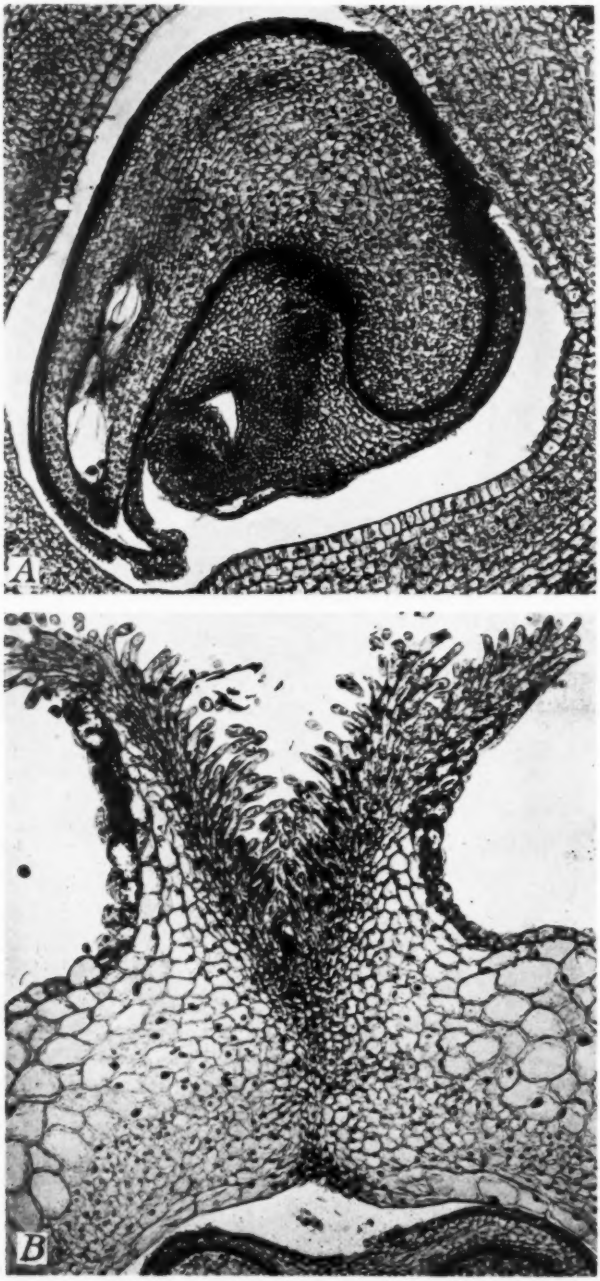
Favorsky (*3*) attempted to germinate beet pollen on artificial media, obtaining, after numerous trials, best growth on sugar agar of high concentration. Only pollen from old anthers developed pollen tubes, and even then not more than 30 percent of the pollen germinated. Fresh pollen from dehiscing anthers showed scant germination, while mature pollen from unopened anthers did not germinate at all.

In the writers' experiments, carried on in New Mexico and Colorado, pollen was found to germinate easily and abundantly on culture media containing 1½ percent agar and 40 percent sucrose (pl. 2, *A*). The optimum germination temperature was about 32° C., which corresponds closely to the prevailing day temperatures during the blooming period. Beet pollen may germinate almost immediately, the tubes attaining a length of about 100 microns during the first 2 hours. The growth increment is uniform, though a small number of pollen tubes are commonly well in advance while some few lag behind and cease growth altogether. This differential rate is even more pronounced in young and in very old pollen. Collections made during the morning hours germinated almost as readily and abundantly as old pollen, thus weakening a popular belief that selfing of beet flowers may be hindered through an enforced rest period in freshly shed pollen. These tests showed also that there is a great variation in the germination percentage of pollen of different flowers; in fact, the pollen of some flowers failed to germinate at all.

Favorsky (*3*), who even under optimum conditions obtained relatively poor germination, tried to arrive at a satisfactory explanation of this condition. He made an extensive study of pollen development and found that a large percentage of pollen degenerated in the later stages of its development. This degeneration seemed to affect all the grains of an anther, and the manner of degeneration frequently varied. He noted particularly that the pollen of otherwise normally stained preparations appeared very dark and that additional destaining resulted in a uniform loss of color without differentiation. The nuclei of such grains were crescent-shaped with frequently only the nucleolus showing. Often the pollen grains appeared quite empty and more or less collapsed, having the appearance of an indented rubber ball. Although partial and even complete degenera-



A, Germinating pollen on artificial medium containing 1½ percent agar and 40 percent sucrose. × 200. *B*, Degenerated and normal pollen; in the latter the sperms and the tube nucleus are visible. × 1,050. *C*, Sterile pollen with nucleus undivided. × 1,050. *D*, Drawing of mature pollen grain showing sperms, tube nucleus, and pollen grains. × 2,400. *E*, Longitudinal section through tip of anther showing degenerated pollen. × 510.



A, Fertilized egg with two endosperm nuclei, one of which is near the egg while the other is in the caecum. × 107. *B*, Longitudinal section of pistil; the stigma lobes are partly expanded. Note the stigma papillae. The latter as well as the epidermal cells of the style contain numerous protein crystalloids. × 120.

tion of anthers had been noted (*2*), observations on general degeneration phenomena of sugar-beet pollen had not been made, and therefore the data of Favorsky (*3*) are of special interest to the writers. Degeneration stages such as he described are illustrated in plate 2, *B*, *C*, and *E*. Such abnormal pollen was found in almost all anthers, and in some flowers degeneration was complete. This rather frequent occurrence of abnormal pollen, while at times perhaps seriously affecting autogamy in beets, should be of little significance in seed production, since relatively enormous masses of pollen are available during the height of the blooming period.

Beet pollen may remain viable for a long period; in fact, the researches of Kharechko-Sawitzkaja (*5*) indicate that some pollen will germinate after 50 days if kept at low temperature and low humidity. Under field conditions in Colorado the viability of pollen does not extend beyond a day, since beet pollen cannot stand wetting by dew. Where pollen is exposed to the rays of the high-altitude sun the viability seems to depend on the nature of the substratum on which the pollen from dehiscing anthers lodges. Thus, pollen collected on shallow glass dishes, paper trays, and detached beet leaves lost its vitality often in less than 3 hours, whereas pollen resting on living beet leaves retained an undiminished germination energy during the course of a day and the failure to germinate the next morning was probably occasioned by the precipitation of moisture on the leaves.

GROWTH OF THE POLLEN TUBE

The style of the sugar-beet flower is very short and terminates with a three-lobed stigma that is closed but begins to open approximately 5 hours after anthesis. The stylar canal is open and densely clothed with papillate cells. Similar cells constitute the epidermis of the stigma lobes (pl. 3, *B*) which become covered with a sweet secretion, an ideal medium for pollen-tube growth. The plastids of the stigmatic papillae contain protein crystalloids, and similar bodies are found grouped around the nuclei of the cells forming the inner lining of the ovary cavity. Favorsky (*3*) found, in addition, protein crystals in the epidermis of the sepals and stamens. These bodies are hexagonal, thereby differing from the crystalloids of the stigmatic papillae as seen in plate 2, *E*.

Pollen was thought to germinate only on open stigmas (*1*), but some observations by Van Heel (*4*) and the germination studies reported above indicate that some pollen at least will germinate hours before the stigma lobes begin to expand.

Beet pollen germinates with a single pore. The pollen tube grows on the surface of the stigma between the loosely connected epidermal papillae. After penetrating the short stylar canal it extends along the inside of the ovary wall until it reaches the micropyle of the ovule, and thence between the integuments and the intervening layers of specialized nucellar tissue. The cells of the nucellus which are in direct line with the integumental canal are narrow, elongated, and rich in cytoplasm, offering seemingly but little resistance to the growing pollen tube.

The number of pollen grains germinating on an open stigma is large, although much pollen degenerates before anthesis and many pollen tubes grow down the stylar canal into the cavity of the ovary.

Sections through 1-day-old flowers show a tangled mass of pollen tubes near the micropyle, the tips of some of them already in the integumental canal (pl. 4, *E*). Oksijuk (*6*) reports that he rarely found pollen tubes in the ovary cavity. This may be true of material fixed in Carnoy's solution, but in chromoacetic formalin the tubes as a rule stand out conspicuously. The number of pollen tubes growing down the micropyle varies and as a rule more than one reaches the embryo sac. During early pollen-tube growth the three nuclei remain in the pollen grain but move into the tube only after the latter has attained a given length. The tube nucleus only slightly precedes the sperms, contrary to the findings of Oksijuk (*6*), who reports that the generative nuclei lag some distance behind the vegetative nucleus.

Upon entry in the embryo sac the pollen tubes apply themselves close to the egg. Their tips are usually dilated and contain the two sperms embedded in densely granular cytoplasm (fig. 2, *D*). The sperms are slightly rounded and have granular chromatin, thus changing from their former elongated, slightly wavy form in the tube (pl. 4, *C*).

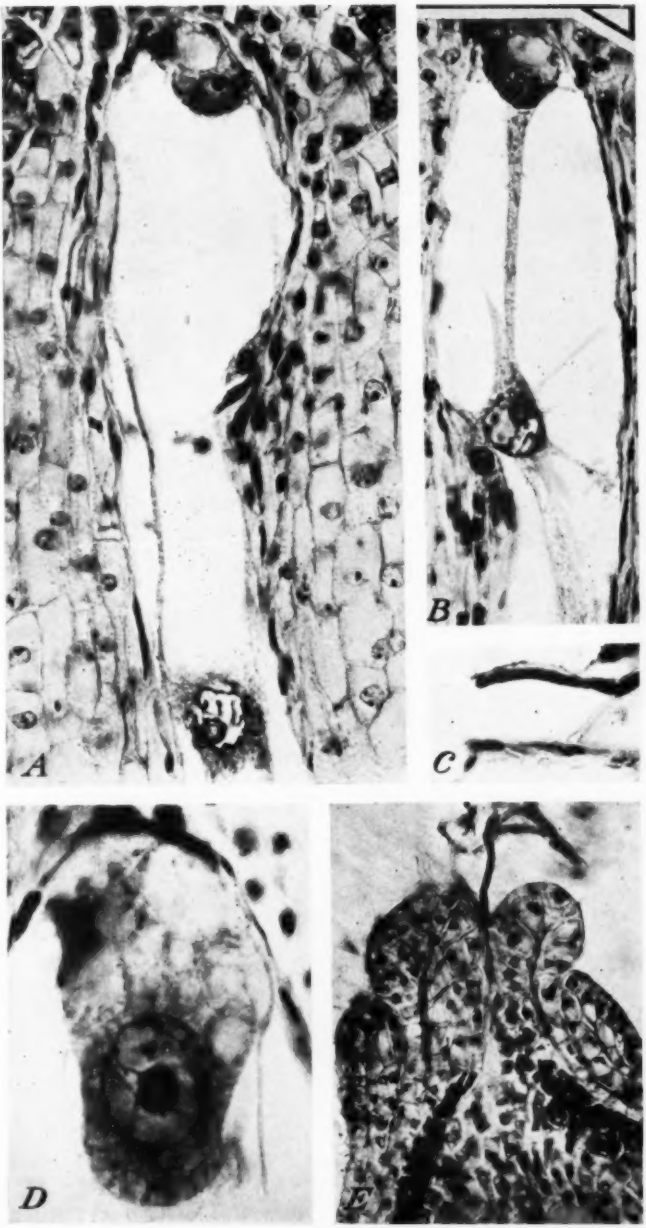
FERTILIZATION

ASSOCIATED PHENOMENA

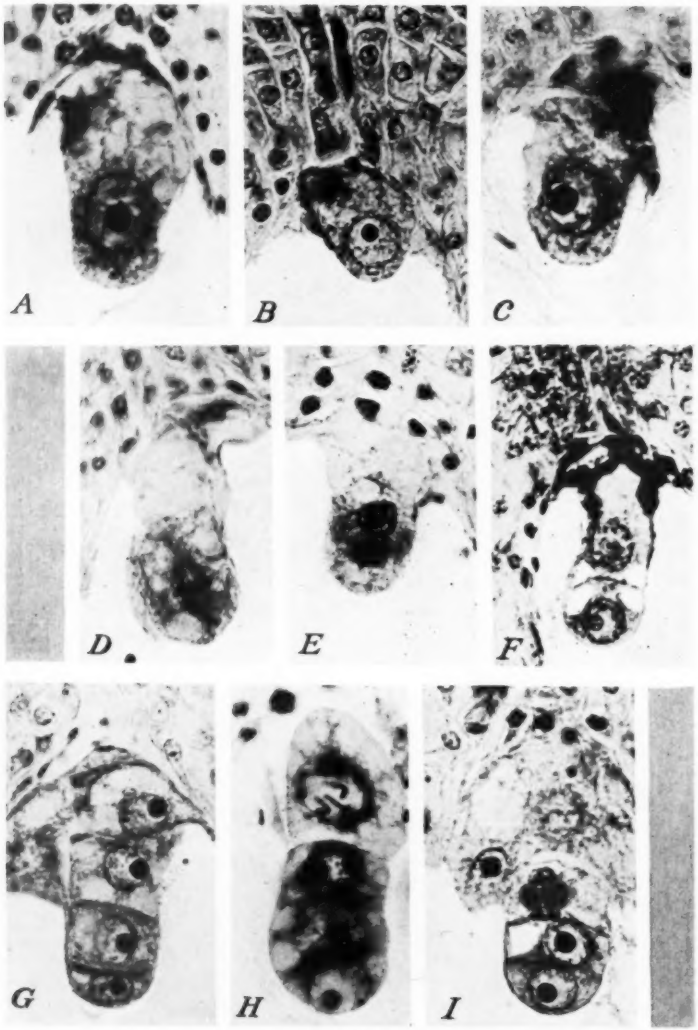
While sperms were observed inside the egg cell in a number of preparations, a study of over 500 fertilization stages failed to show actual fusion between sperm and egg nucleus, which, it appears, is accomplished very rapidly. As a characteristic accompaniment of fertilization there are certain dark-staining round or oval bodies in the vicinity of the egg. These bodies often resemble in form and structure the generative nuclei, and their frequent position in the proximal end of the supernumerary pollen tubes suggests their sperm nature. Most frequently they occur in pairs or double pairs flanking the egg and occupying the place of the degenerating synergids (pl. 5, *B*, *C*). Similar structures have been observed coincident with fertilization in other plants (*7*). They have been called X-bodies by different writers who interpreted them as the nuclei of the synergids, the degenerated cytoplasmic remains of the male cells, or as the tube nucleus. In the sugar beet these bodies frequently have typical sperm structure even though in older material they will have lost their inner differentiation and appear when stained as homogeneous black bodies. Usually they differ conspicuously from the nuclei of the synergids and only in some preparations might their degenerated remains be interpreted as a part of these so-called X-bodies. These bodies, whatever their nature, persist for a long time and may be easily identified in material containing 10-celled embryos.

The newly fertilized egg contains two nucleoli, but this condition persists for only a very short time, since only one nucleolus is present after the first division of the fusion polar.

The synergids begin to degenerate soon after anthesis and before the pollen tube has penetrated into the cavity of the ovary. The first indication of a change is a slight yellow discoloration at the base of the synergids, followed by a disappearance of the large vacuole below the nucleus and a general telescoping of the cell, which takes on the shape illustrated in figure 2, *B*. The advance of the pollen tube to the proximity of the egg usually causes the destruction of one



A, Embryo sac with fertilized egg and dividing fusion polar. × 675. *B*, Embryo sac with dividing fusion polar just above the antipodals. × 675. *C*, Pollen tubes, the lower one containing two sperms. × 787. *D*, Mature fertilized egg. × 1,380. *E*, Pollen tube entering micropyle. × 397.



A, Fertilized egg. × 824. *B*, Fertilized egg with two X-bodies. × 600. *C*, Fertilized egg with two X-bodies. × 960. *D*, Mitosis in fertilized egg. × 803. *E*, Telophase in first division of fertilized egg. × 824. *F*, 2-celled embryo. × 664. *G*, 4-celled embryo. × 960. *H*, 4-celled embryo with basal cell dividing. × 1,120. *I*, 4-celled embryo with two lower cells dividing simultaneously. × 960.

of the synergids, the second disappearing before the egg divides to form the embryo. In case of delayed pollination or fertilization the synergids remain unchanged for several days, but if an embryo fails to develop, they will degenerate prior to any other nuclei in the embryo sac.

THE TIME FACTOR

Since stained preparations of material fixed in Carnoy's solution show pollen tubes indistinctly, several hundred flowers were killed in a modification of Nawaschin's solution. The pollen tubes thus fixed stood out rather sharply and could be easily located in the cavity of the ovary and the micropylar canal.

Material fixed 22 hours after anthesis showed pollen tubes in the ovary cavity in all but one flower. In approximately 50 percent of the cases fertilization of the egg had already occurred. The fusion polar was in the caecum just below the antipodals and evidently not yet fertilized. Material fixed 24 hours after anthesis showed that all eggs were fertilized and each contained two nucleoli. The fusion polar was binucleolated in some flowers; in others there were already present two endosperm nuclei. Material fixed 28 hours after anthesis showed all flowers fertilized, but the eggs contained, at this stage, only one nucleolus. There were, without exception, two endosperm nuclei in each embryo sac.

It appears from this study that fertilization takes place approximately 1 day after anthesis; and since pollen germinates as early as 2 hours after the anthers dehisce, the time factor in fertilization is theoretically about 20 hours. However, in computing this factor it must be remembered that although fresh pollen may germinate within 2 hours after shedding, older and dried-off pollen germinates even sooner, and when it is applied to open instead of closed stigmas the time is likely to be still further reduced. Both fresh and dried-off pollen may germinate on unopened stigmas, but the growth on the unopened stigmas is slow, enabling the tubes from pollen applied several hours later to partly open stigmas to reach the embryo sac not far behind, perhaps even in advance of, those not so advantageously placed. This fact might have an important bearing on the question of cross-pollination, since the first germinating pollen belongs most likely to the same flower, while in free-blooming beets hybridization is the rule.

While this series of fertilization studies is based on material collected between June 19 and June 23, 1932, preparations from the previous season showed fertilization stages as early as 6 hours after anthesis. The data were, on the whole, not so uniform as those obtained in 1932, and showed much individual variation. However, the fact that in some beets numerous fertilization stages occurred as early as 10 hours after anthesis cannot be considered an accident, but rather argues that pollen-tube growth proceeds more rapidly in the flowers of some plants than in those of others. Oksijuk (*6*) observed fertilization as early as 3½ hours after the flower opened, while 7 hours after anthesis about two thirds of them were fertilized. Since he does not describe his methods of marking the flowers, it appears, in view of the writers' data, rather doubtful whether fertilization normally takes place in so short a time after the pollen is shed.

Favorsky (*3*) tells of finding young embryos in tightly closed buds; but since fertilization of such flowers seems to be out of the question, he explains their presence as due to nucellar budding, a phenomenon that he had occasion to observe a number of times.

Weather conditions may and probably do have an influence on the time factor in fertilization. The month of June was warmer in 1931 than in 1932, and likewise the time required for the pollen tube to reach the embryo sac was shorter in the warmer month.

POST-FERTILIZATION PERIOD

The egg, unlike the primary endosperm nucleus, does not divide immediately after fertilization but undergoes a rest period, the length of which probably depends on both external factors and the genetic constitution of the plant. During this rest period general growth processes in ovule and embryo sac continue, while in the nuclei of the latter both progressive and retrogressive differentiations take place.

Measurements showing the progressive increase in the size of the ovule are given in table 2. The ovule at the time of anthesis has a long horizontal diameter of approximately 0.5 mm. It enlarges rapidly, having increased its linear dimensions approximately 40 percent at the time of fertilization (compare pl. 1, *A*, and pl. 3, *A*), and is almost fully grown, while the embryo is still very small. This rapid growth of the ovule during the early post-fertilization period is in harmony with the organization of the seed, which has a perisperm as a storage tissue. The endosperm has only a temporary existence and is consumed by the developing embryo.

Concomitant with the enlargement of the ovule is an increase in size of the embryo sac. The latter not only gains in length but broadens as well, especially during the first days after fertilization.

TABLE 2.—*Increase in size of ovule and embryo sac after anthesis*

Time after anthesis	Flowers examined	Longest horizontal diameter of ovule	Length of embryo sac	Width of embryo sac
	Number	μ	μ	μ
Check [a]	9	549	76	44
2 to 3 hours	63	569	82	48
4 to 5 hours	59	686	84	44
6 to 10 hours	27	686	80	43
17 to 20 hours	25	739	100	52
1 day	63	762	92	48
2 days	41	893	128	64
3 days	20	1,258	172	104
4 days	21	1,320	196	
5 days	15	1,918		
6 days	6	2,024		
10 days	3	2,464		
Mature seed	4	2,485		

[a] Examined at time of anthesis.

More conspicuous, however, than the enlargement of the embryo sac is the growth of the caecum, which is more or less an extension of the former below the antipodals. At the time of anthesis it is slightly shorter than the embryo sac but rapidly enlarges into a horseshoe-shaped cavity. This cavity is quite narrow but broadens somewhat at its distal end, which is easily recognized by an accumulation of

cytoplasm and free endosperm nuclei. The cells of the central nucellar tissue, which upon disintegration give rise to the caecum, are narrow and more elongated than the neighboring ones, and, according to Favorsky (*3*), they stain specifically with Pianese some time prior to disintegration. Soon after anthesis there appear in this specialized nucellar region narrow cavities which, as tissue breakdown spreads, unite into one common opening. This mode of formation is at times replaced by progressive tissue breakdown which has its beginning at the base of the embryo sac. In either case, however, the caecum is fully developed when the egg divides to form the embryo.

Both synergids and antipodals degenerate and disappear after fertilization. The synergids begin to degenerate soon after anthesis and have changed noticeably in appearance even before the pollen tube reaches the embroyo sac. The pollen tube usually causes the

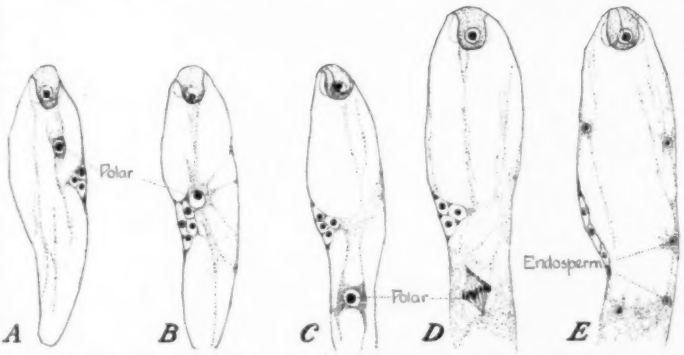


FIGURE 4.—Diagrammatic drawings illustrating three characteristic positions of the fusion polar in the embryo sac: *A*, Polar above antipodals; *B*, polar opposite antipodals; *C*, polar below antipodals in the caecum; *D*, first division of fusion polar; *E*, marginal endosperm nuclei, the egg about to divide. (Compare with table 3.)

destruction of one of the synergids, and the complete degeneration of the other occurs soon afterwards.

The life of the antipodals, however, does not terminate with the advent of fertilization. There is usually an increase in their number, and the nuclei retain a normal structure for a number of days after fertilization, but finally, with the progressive destruction of nucellar tissue and the widening of the embryo sac, they become at first flattened and then degenerate and disappear.

The fusion polar or primary endosperm nucleus divides immediately after fertilization. Its position varies in the embryo sac (fig. 4). In young closed flowers, it is located midway between egg and antipodals, but as the flowers open, the polar usually moves to the lower end of the sac and thence into the caecum. A study of table 3, which gives the percentage distribution of the polar in relation to the antipodals, shows the fusion polar in the caecum below the antipodals in the majority of cases. Its position in the embryo sac, at a given age of the flower, does not appear to be bound up with the genetic constitution of the plant, although occasionally individuals will be found in which the fusion polar occupies one and the same

position in all the flowers of the same age. At the time of fertilization the fusion polar is normally in the caecum. Occasionally it is found near the antipodals or even above them, but whatever its position at this time, the daughter nuclei resulting from the first division will separate, one moving farther down in the caecum and the other in the opposite direction toward the egg. The first division of the polar is followed by a second and a third division, so that at the time the egg ends its rest period there will be present in the embryo sac a number of endosperm nuclei.

TABLE 3.—*Position of fusion polar relative to the antipodals, mitosis in fusion polar and distribution of endosperm nuclei*

Time after anthesis	Number of flowers examined	Percentage distribution of fusion polar relative to the antipodals, mitosis in fusion polar, and appearance of endosperm nuclei [a]				
		A	*B*	*C*	*D*	*E*
2 to 7 hours	376	18	11.9	64		
10 to 20 hours	75	10.6	9.7	40	4	36
24 hours	334	6.2	8.3	31.7	4.3	51.1

[a] Letters correspond to those in figure 4.

EMBRYO DEVELOPMENT

FORMATION

After the fertilized egg has undergone a rest period it divides horizontally (pl. 5, *D*, *E*). Of the two daughter cells thus formed (pl. 5, *F*, and fig. 5, *A*), the lower one soon divides again transversely (fig. 5, *B*), and this is followed by a division of the upper or apical cell (fig. 5, *C*). These 2 divisions result in a tetrad of superimposed cells (pl. 5, *G*), of which the upper 3 form, by subsequent divisions, the embryo proper, while the basal cell gives rise to the hypophysis and the suspensor. Oksijuk (*6*) believes that the basal cell of the 2-celled embryo does not take part in the formation of the embryo, but that it enlarges and assumes the character of a haustorial cell. A study of plate 5 and figure 6, which give the sequence of cell divisions in the fertilized egg, fails to substantiate his contentions, and hence his criticism of the mode of embryo formation as given in an earlier publication by the senior writer (*2*) is, in the light of these new investigations, entirely unjustified.

Each element of the tetrad divides once to form the 8-celled embryo, but whereas in the formation of the tetrad a sequence pattern was rigidly adhered to, the time and place factors do not play such a vital role in the formation of the octant. The 4 cells of the tetrad are for convenience designated *a*, *b*, *c*, and *d*; *a* representing the apical and *d* the basal cell. Cell *d* usually divides first (pl. 5, *H*, and fig. 5, *D*), whereby the direction of the division wall is always transverse. Cell *c* of the tetrad divides later but sometimes simultaneously with *d*. The direction of the dividing wall may be either transverse or vertical (pl. 5, *I*, and fig. 5, *E*, *F*). Cells *a* and *b* of the tetrad may divide simultaneously (fig. 7, *B*), or, as is more often the

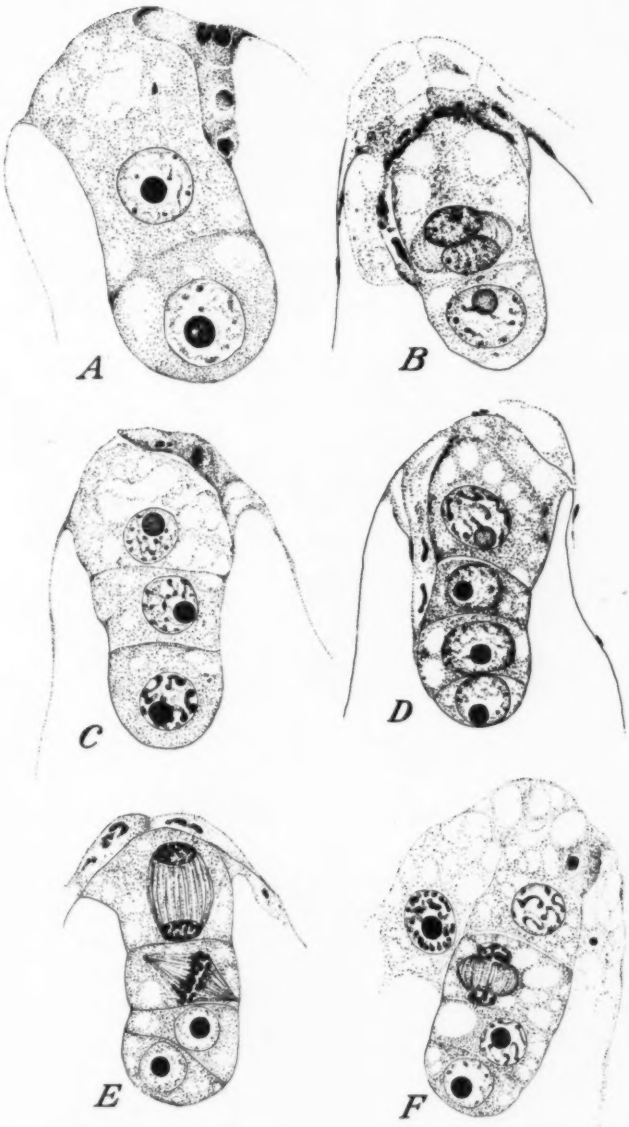


FIGURE 5.—*A*, 2-celled embryo with X-bodies still visible at the periphery of the basal cell, × 1,380; *B*, 2-celled embryo with basal cell dividing, × 1,050; *C*, 3-celled embryo with upper cell dividing, × 1,050; *D*, 4-celled embryo with basal cell dividing, × 1,050; *E*, 4-celled embryo with the 2 lower cells dividing, the spindle of the penultimate cell vertical to the long axis of the embryo, × 772; *F*, same as *E*, but penultimate cell divides transversely instead of by a vertical wall, × 772.

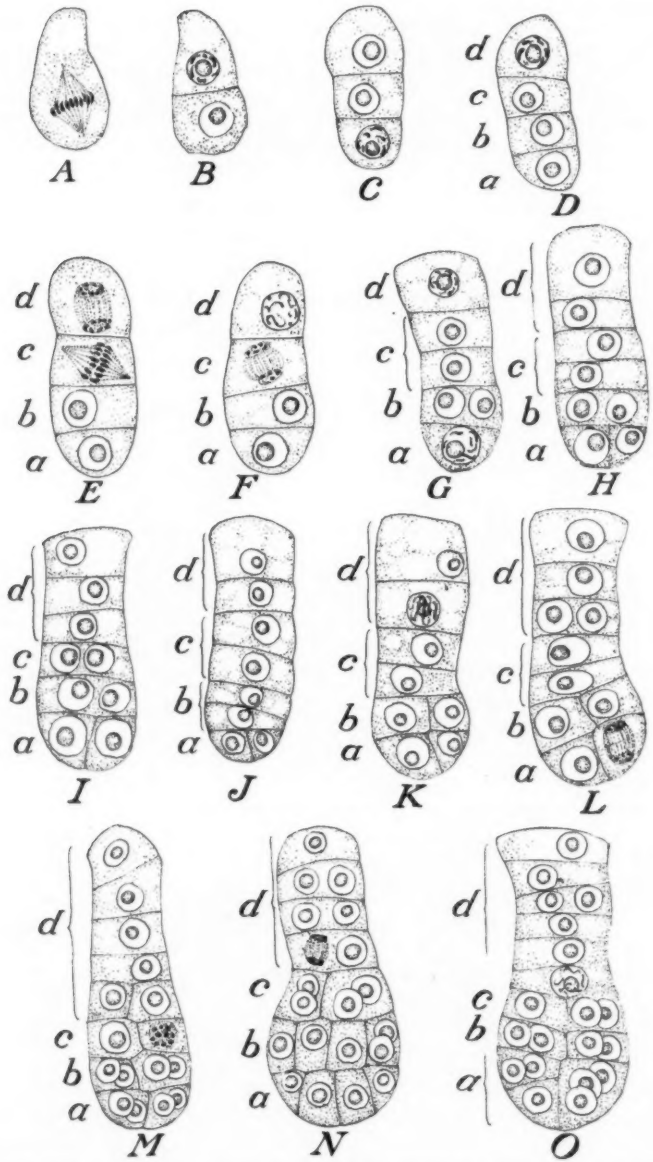
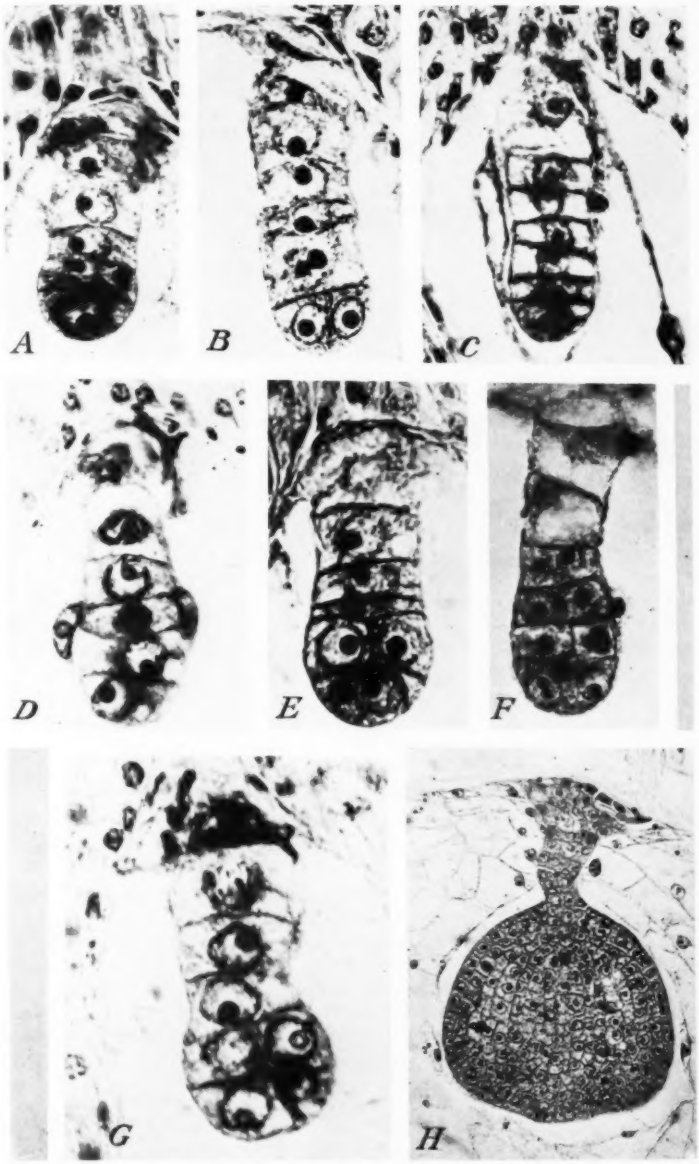


FIGURE 6.—A series of 15 diagrammatic drawings showing the sequence of cell division in the formation of the young embryo.



A, Similar to figure 7, *A*. × 850. *B*, Similar to figure 7, *D*. × 722. *C*, A 7-tiered octant. × 722. *D*, Simultaneous mitosis in cells of a 6-tiered octant (for detail see figure 8, *A*). × 731. *E*, Similar to *D*, except that cells are not dividing. × 731. *F*, 16-celled embryo. × 595. *G*, Octant with penultimate cell dividing. × 867. *H*, Globular embryo surrounded by cellular endosperm. × 212.

case, one may divide before the other (fig. 7, *A*, and pl. 6, *A*). Cell *a* always divides by a vertical wall, but *b* may divide either transversely (fig. 7, *C*, *E*) or vertically (pl. 6, *A*, *B*, *E*, *F*, and *G*, and fig. 7, *A*, *D*, *F*), the latter mode of division being the more frequent. Thus, depending on the direction of the division walls in *b* and *c*, the 8-celled embryo is either 5-, 6-, or 7-tiered (pl. 6, *C*). The formation of the tetrad in the sugar beet tallies with the development of the proembryo in *Chenopodium*, as described by Souèges (*8*), but the subsequent divisions in the latter differ in that cell *B* always and cell *c* usually divide by vertical walls so that the octant is commonly 5-tiered.

The 16-celled embryo (fig. 8, *D*) is formed by the division of the elements of the 8-celled embryo. The direction of the division walls will largely be governed by the behavior of the cells of the tetrad. If the resulting octant is 5-tiered, the 2 superimposed daughter cells of *d* divide again horizontally to form a row of 4 cells. Cell *c*, which divided by a vertical wall in the formation of the octant, now divides transversely, while the daughter cells of *a* and *b* divide again vertically. The resulting 16-celled embryo has 8 tiers with the upper 2 forming in cross section each a quadrant. If either *b* or *c* of the 4-celled embryo divided transversely the resulting octant would be 6-tiered (pl. 6, *D*, and fig. 8, *A*, *C*), and in order for the 16-celled embryo to have the normal number of 8 tiers the division walls of the 6-tiered octant would differ somewhat from those of the 5-tiered octant. If the first division of *c* were transverse (fig. 8, *B*) the second division would be vertical, whereby the normal tiered arrangement of the 16-celled embryo would be established. In case *b* divided transversely in the formation of the octant, equalization in the normal number of tiers would not be attained in the embryo until after a series of subsequent divisions. The same holds true for the 7-tiered octant. An examination of figure 6 shows other irregularities in the sequence of cell division which give the impression that, after the formation of the quadrant, no strict law governs the sequence of cell division. However, a certain balance seems to be maintained so that the final product is remarkably uniform.

THE TIME FACTOR

Two-celled embryos have been observed as early as 24 hours after anthesis, but in the majority of cases the egg does not divide until 36 hours after the flower has opened. It has previously been shown that fertilization is effected within a day after anthesis or even sooner, so that the rest period of the egg equals the time necessary for the pollen tube to reach the embryo sac. This view, substantiated by a large number of observations on carefully marked material, must needs take the place of an earlier opinion of the senior writer that the fertilized egg divided immediately or after a short period of rest. There must likewise be a revision in the views of other writers, who, although correctly assuming a rest period, find 2-celled embryos about 4 days after anthesis, whereas normally most eggs have already divided 2 days after the flowers open.

The time factor in the early embryogeny of the sugar beet is illustrated in table 4. It will be seen that early growth and differentiation are extremely slow. Eight-celled embryos are most plentiful

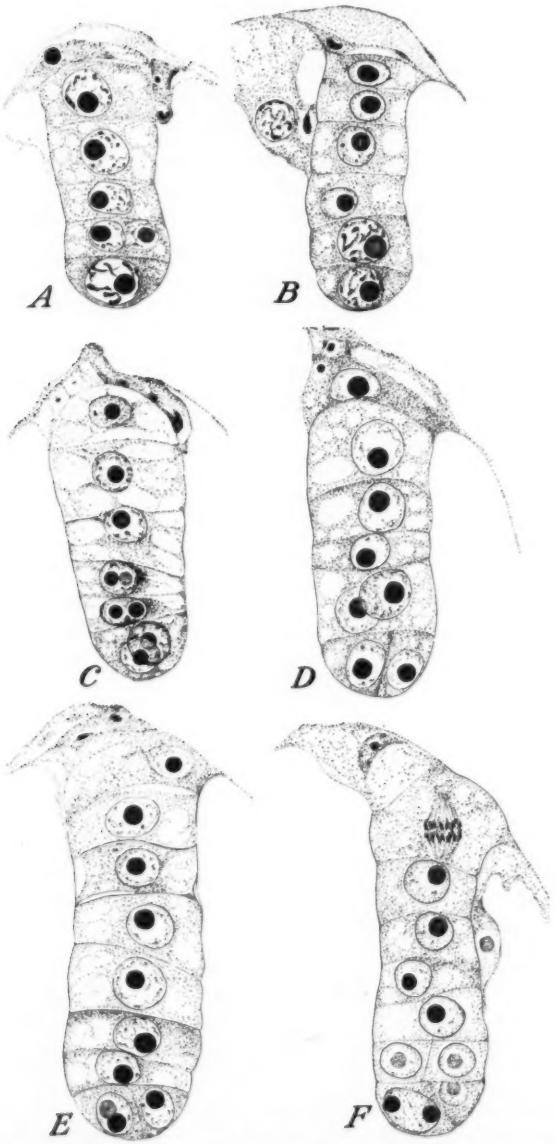


FIGURE 7.—*A*, 5-tiered embryo with the cell next to the apical divided in advance of the apical cell, × 1,260; *B*, 6-tiered embryo showing simultaneous division in the 2 apical cells; *C*, a later stage; while the apical cell is dividing vertically, the cell next to it has divided horizontally, × 1,260; *D*, similar to *C* except that the cell next to the apical is dividing by a vertical wall, × 1,260; *E*, a later stage of *C*, × 1,260; *F*, a stage differing from the preceding one principally in that the cell next to the apical has divided vertically, × 1,260.

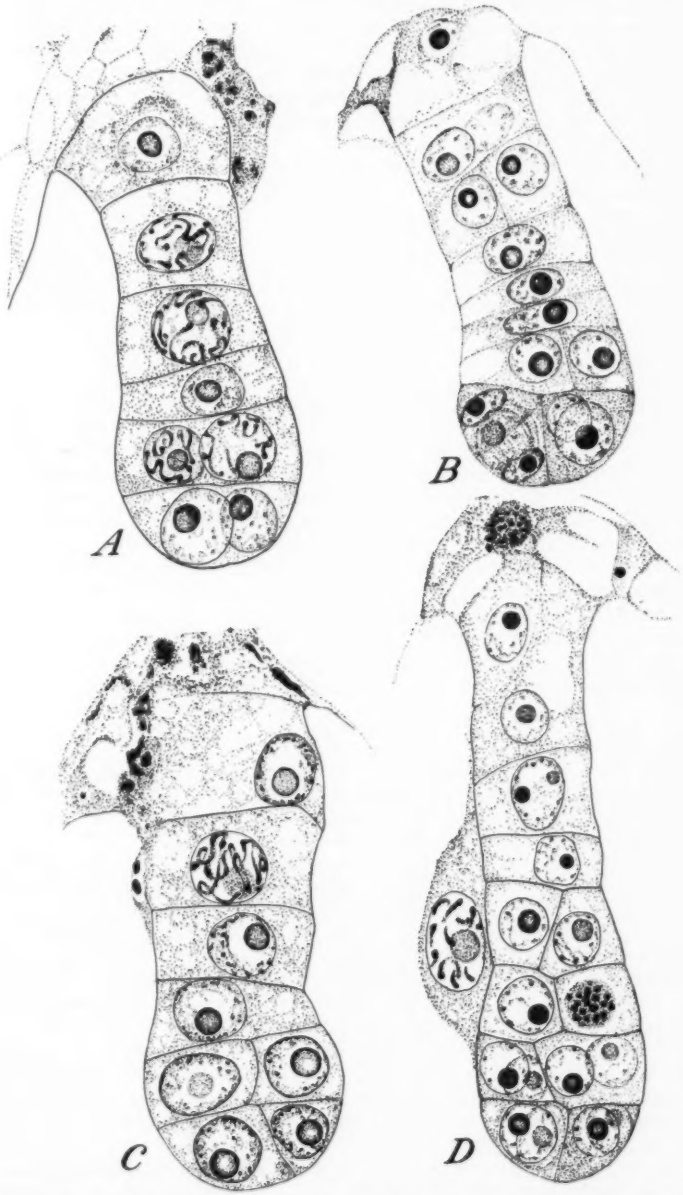


FIGURE 8.—*A*, Detailed drawing of plate 6, *D*, × 1,530; *B*, cell division in the octant, × 1,190; *C*, detailed drawing of plate 6, *G*, ×1,530; *D*, 16-celled embryo, with a laterally attached endosperm nucleus, ×1,190.

from 2½ to 3 days after anthesis, while club-shaped embryos of 16 cells or more dominate the picture about 1 day later. The globular form (pl. 6, *H*) appears about the fifth day, and differentiation of the cotyledons a day or two later. Growth continues very slowly up to the eighth or ninth day, and thence rapidly, so that the embryo is mature by the twelfth or thirteenth day.

TABLE 4.—*The time factor in embryo development*

Time after anthesis (days)	Flowers examined	Fertilized eggs	Eggs dividing	2- to 3-celled embryo	4- to 5-celled embryo	6- to 8-celled embryo	16-celled embryo	Older embryo
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1	334	86.5	.....	9.7	2	1.4	.....	.....
2	137	27	8.7	24	22.5	15.3	2.1	.....
2½	53	9.4	.....	7.4	24.4	60.2	.....	.....
4	61	.....	.....	.....	3.1	11.3	8.1	77

Concomitant with the differentiation of the embryo is the development and subsequent disappearance of the endosperm. Its development is initiated with the fertilization of the fusion polar, which, unlike the egg, does not pass through a rest period. The endosperm nuclei migrate to the periphery of the sac, where they become embedded in cytoplasm. At the termination of the rest period of the egg there are present in the embryo sac from 4 to 6 nuclei and not infrequently an even larger number. Mitosis in the endosperm nuclei is simultaneous. The period of free nuclear division continues to the fifth day, when the embryo has attained a globular form. The nuclei then become invested by walls, and gradually the entire cavity of the embryo sac becomes filled with a delicate tissue, which, however, is again dissolved and the contents absorbed by the growing embryo.

SUMMARY

This investigation is based on a study of about 3,000 sugar-beet flowers collected during the month of June 1931 and 1932 at Rocky Ford, Colo. The flowers were marked at the time of anthesis, and collections were made at definite intervals.

The stigma of the young beet flower is closed. It begins to open in midafternoon, approximately 7 hours after anthesis, and expansion is completed within 24 to 36 hours; occasionally the stigma begins to open sooner and the lobes are fully expanded a few hours later.

The embryo sac has the normal nuclear complex. The fusion polar migrates into the caecum sometimes even before anthesis. The caecum begins to form before the flower opens, and its development is completed soon after the egg is fertilized.

The ovary with its ovule and embryo sac has usually a normal structure. There have been observed the following abnormalities: 2 ovules within an ovary; 2 eggs within an embryo sac; abnormal elongation of nucellar tip and inner integument; partial or complete suppression of the embryo sac.

The pollen of a given flower is fairly uniform in size, since grains of extreme dimensions are relatively few. Pollen grains of different selections have a different average diameter, but their plotted measurements rarely give a bimodal curve as claimed by Oksijuk (*6*) for

his material. A great deal of pollen degenerates, but, even so, many pollen grains germinate and grow down the stylar canal into the cavity of the ovary, and as a rule more than one pollen tube reaches the embryo sac. The time necessary for the pollen tube to reach its destination is about 20 hours. In some flowers this time factor is reduced to 10 hours or even less.

Fertilization is accomplished soon after the pollen tube reaches the embryo sac. Accompanying this process are certain dark-staining bodies which usually flank the egg in pairs and constitute in most cases supernumerary sperms.

The synergids begin to degenerate before fertilization, but the antipodals very often increase in number and do not disappear until embryo development is well under way.

The fusion polar divides without undergoing a rest perid. At the time of fertilization there are usually present from 4 to 6 endosperm nuclei.

The egg undergoes a rest period which approximately equals the time it takes the pollen tube to reach the micropyle. Two-celled embryos are found commonly 36 hours after anthesis and occasionally even sooner. In the subsequent development of the embryo, growth is at first very slow. Differentiation of the cotyledons is noticeable in about 6 days, or somewhat later than the appearance of cellular endosperm, and the embryo is mature from 12 to 14 days after anthesis. The first divisions of the egg are transverse and result in a tetrad of superimposed cells, of which the upper three form the embryo proper, while the basal cell gives rise to the hypophysis and the suspensor.

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CYTOLOGICAL FEATURES OF NICOTIANA GLUTINOSA HAPLONTS[1]

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This paper represents the sixth in a series, the first five numbers of which appeared in the University of California Publications in Botany (*1, 14, 22, 23, 24*).[3] The investigation on which this paper is based was begun while the writer was a research associate in the department of botany at the University of California, and was completed after he joined the staff of the Bureau of Plant Industry, United States Department of Agriculture.

INTRODUCTION

Nicotiana glutinosa L. has been grown in pure line for over 25 years in the botanical gardens of the University of California. It is one of the most distinct species of the genus and is strictly monomorphic. The leaves are small, cordate, long-petioled, and extremely glandular. The flowers, which are light yellow tinged with deep red, are short-cylindrical at the base, but above the base they expand abruptly into an irregular, obliquely one-sided funnel. The limb is somewhat bilabiate. The stigma and anthers are connivent just under the middle lobe of the upper lip. Although the species is included in the *N. rustica* L. section of the genus (*7*) its zygomorphic flowers show a close relationship to *N. tomentosa* Ruiz and Pav. of the *N. tabacum* L. section. It possesses 12 pairs of chromosomes.

Three *Nicotiana glutinosa* haplonts have been found among the plants in the botanical garden. Although the first of these was discovered among 24 seedlings that were subjected to X-radiation, the variation was apparently spontaneous and not the result of the treatment (*11*). The two other haplonts, 30.179-9 and 30.180-49, occurred in two F_3 populations of 50 plants each, grown from X-rayed seed during the spring of 1930. The parents of both of these populations were selected as having lower vigor and fertility. It is probable that these two haplonts were due to haploid parthenogenesis stimulated by an increased incompatibility of male and female gametes.

Except for considerable reduction in all the parts, the haplonts are normal in appearance. The flowers are slightly lighter in color, and the anthers and stigma, although noticeably smaller, are essentially normal. The plants are self-sterile and produce no seed when pollinated with diplont pollen. Photographs of diploid and haploid plants are shown in figures 1 and 2.

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[2] The writer is much indebted to Dr. T. H. Goodspeed, professor of botany, University of California, for the experimental material and for the opportunity to pursue the study, and to Dr. I. E. Webber, of the Division of Blister Rust Control, Bureau of Plant Industry, for helpful suggestions in regard to histology and for aid in the preparation of the manuscript.

[3] Reference is made by number (italic) to Literature Cited, p. 866.

Haplonts in general exhibit many interesting cytological phenomena, some of which may serve as a basis for the interpretation of certain complex processes of meiosis in diplonts and hybrid plants. The early prophase stages of haplonts furnish evidence regarding the origin and nature of the double chromosomes seen in the later stages of the

FIGURE 1.—*Nicotiana glutinosa: A*, Diplont 30.180-48; *B*, haplont 30.180-49.

diplont. Since, in general, haplonts form univalent chromosomes in the first metaphase, many irregularities occur during the remaining stages of sporogenesis. Such irregularities as univalent division, fragmentation, and elimination are frequent and exceptionally clear. The formation and behavior of binucleate germ cells and of unreduced gametes and finally the steps of degeneration are likewise frequent and

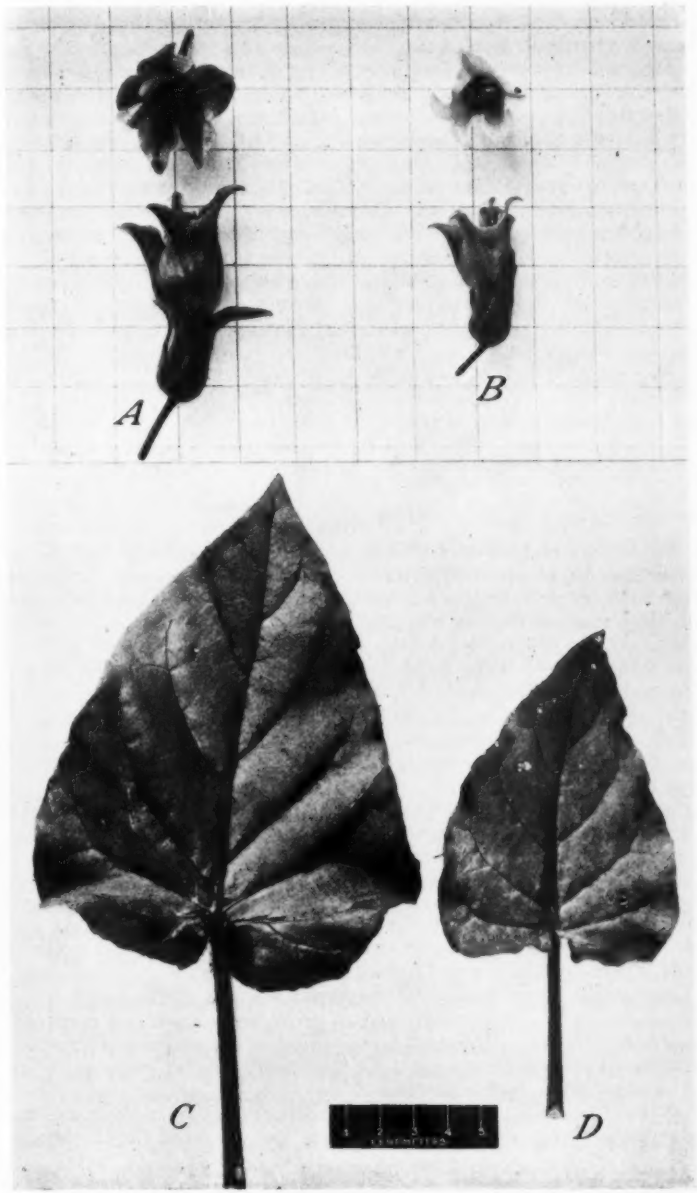


FIGURE 2.—*Nicotiana glutinosa: A*, Typical flowers of diplont 30.180-48, shown against background ruled in centimeters; *B*, typical flowers of haplont 30.180-49; *C*, typical leaf of diplont 30.180-48; *D*, typical leaf of haplont 30.180-49.

clear. Similar products of such irregularities observed in other plants doubtless originated in many cases from phenomena closely related to those occurring in haplonts.

Because of the reduced number of chromosomes in the somatic cells, the root tips are obviously most favorable for the study of chromosome morphology. Moreover, such root tips frequently contain diploid cells and tissues. The formation of diploid areas in haplont roots and comparisons of such areas with tetraploid areas in roots of diplonts are extremely interesting. The cytology of root initials, callus tissues, and cuttings presents evidence as to the nature of diploid areas in haplonts and their relative frequency of occurrence in root and shoot.

Studies in chromosome number and morphology in *Nicotiana* have demonstrated that characteristic chromosome features distinguish certain species. The results for *N. tabacum* (*23*), *N. alata* Link and Otto (*1*, *22*), *N. longiflora* Cav. (*14*), *N. sylvestris* Spegaz. (*24*), and *N. langsdorffi* Schrank,[4] together with the data included herein, indicate that specific chromosome features furnish evidence as to the origin and relationship of species within the genus. It is encouraging that such evidence is accumulating and that it harmonizes closely with the evidence obtained from interspecific hybridization.

MATERIAL AND METHODS

The seeds giving rise to *Nicotiana glutinosa* haplonts 30.179-9 and 30.180-49 and to sister diplonts were germinated in pots. The seedlings were later transplanted into flats; when approximately 6 weeks old, they were set out in the field.

When approximately 16 weeks old, plants 30.179-9 and 30.180-49 were suspected of being haplonts and a few root tips were carefully collected from each plant. Soon after this 30.179-9 was injured and died. When 22 weeks old, 30.180-49 was known to be a haplont, and anther, ovary, and additional root-tip material were collected. At this time the plant was potted, removed to the greenhouse, and 20 stem cuttings were made.

Two weeks later root tips were gathered at random and from marked roots of the haplont. When the plant was 26 weeks old, root tips were similarly collected, and material for a study of root initials was obtained. By this time the roots had become so pot-bound that it was necessary to remove all exposed roots. Subsequent root-tip collections were made when the plant was 28, 30, 35, 37, and 41 weeks old. All exposed roots were removed after the root-tip collection made when the plant was 37 weeks old.

During this period and until the writer last saw the haplont, when it was 1½ years of age, it continued to grow vigorously and to produce numerous flowers. Frequent cytological examinations showed that its shoots remained haploid. As previously stated, the sexual cells of the plant were highly sterile.

Of the 20 cuttings, 13 produced callus tissues over their proximal ends and 7 died. Two of the surviving 13 were used for the study of callus tissue and root initials. In order to stimulate distal callus formation, a fresh cut was made across the apical internode of 5 of the

[4] RAUP, L. J. MEIOTIC BEHAVIOR OF NICOTIANA GLUTINOSA HAPLOIDS. (Thesis, University of California.) [Unpublished.]

remaining cuttings. The cuts on 3 of these were immediately covered with petrolatum. All 5 were kept in a humid chamber and only 1 shoot bearing 2 leaves was allowed to develop on each. In no case did the cuttings form callus tissue at the distal end. The remaining 6 cuttings were allowed to develop, and their root tips and flower buds were subjected to cytological examination. All shoots produced were haploid and remained haploid until the plants died.

Diploid material of *Nicotiana glutinosa* was obtained from plants 27.028-6, 27.028-7, 28.028-53, and 30.005-1. The populations containing these four plants were grown from pure untreated seed.

The cytological observations were made on paraffin material, permanent smears, and acetocarmine smears. All cut material was killed and fixed in the following chromoacetic-formalin mixture:

1 part: 65 cc water, 10 cc glacial acetic acid, and 1 g chromic acid.
1 part: 40 cc commercial formalin and 35 cc water.

Before paraffin infiltration, callus tissues were softened in diaphenol. Paraffin sections were cut 10μ thick and stained in Heidenhain's iron haematoxylin.

Somatic chromosome drawings and measurements were obtained from division figures located in the upper portion of the zone of cell division in the periblem. In this region the chromosomes vary only slightly in size and shape.

Camera-lucida drawings were corrected by direct observation. The drawings are reproduced at the following diameters: Somatic chromosomes and callus cells, at 4,750; pollen mother cells and sporads, at 2,000; and female gametophytes, at 950. No attempt has been made to show the relative levels at which the chromosomes lie.

CHROMOSOME MORPHOLOGY IN HAPLONT AND DIPLONT NICOTIANA GLUTINOSA

The haploid chromosome number of *Nicotiana glutinosa*, recorded by Goodspeed (*9*, *10*) as 12, has been repeatedly confirmed by cytologists. Christoff (*3*) determined and figured the 24 somatic chromosomes of the species. His figures clearly show that the chromosomes of the complex vary but little in size. He found that the somatic chromosomes of *N. glutinosa* are shorter and slenderer than those of *N. paniculata*.

In the present study a large number of favorable somatic metaphase plates were examined in root tips from both haplonts and diplonts. A casual observation of diploid plates (pl. 1, *G–I*) gives the impression that the chromosomes are all of approximately the same size but readily divisible into a group of V-shaped and a group of rod-shaped chromosomes. However, a detailed study of these chromosomes shows slight variations in size and form within each of the two groups.

A close examination of the chromosomes composing the V-shaped group shows that they may be classified under six types (fig. 3). Type 1 comprises two pairs of chromosomes having median constrictions. Each segment of this group of four chromosomes averages 1.80μ in length. Type 2 likewise comprises two pairs of medianly constricted chromosomes. However, the segments of this type average 2.00μ in length. Type 3 comprises one pair of medianly constricted chromosomes, the segments of which average 2.26μ in length. Obviously the chromosomes of the foregoing five pairs are

strictly V-shaped and are very nearly the same in size, which makes it difficult to identify them in diploid plates.

Type 4 comprises two pairs of submedianly constricted chromosomes, each with segments averaging 1.60μ and 2.15μ in length. Type 5 comprises a single pair of submedianly constricted chromosomes. The segments of this pair average 1.80μ and 2.26μ in length. The chromosomes of types 4 and 5, as in the case of types 1, 2, and 3,

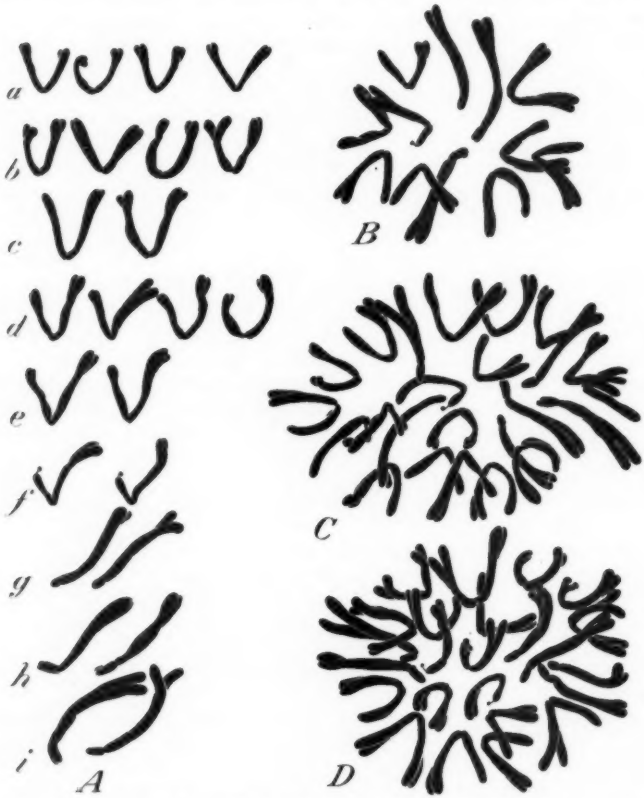
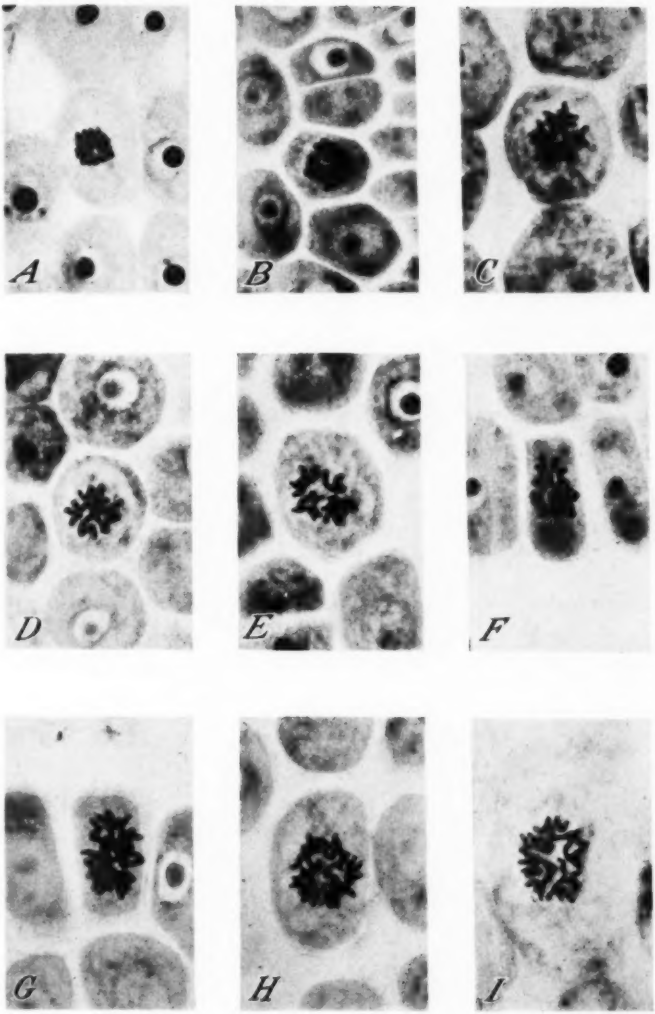


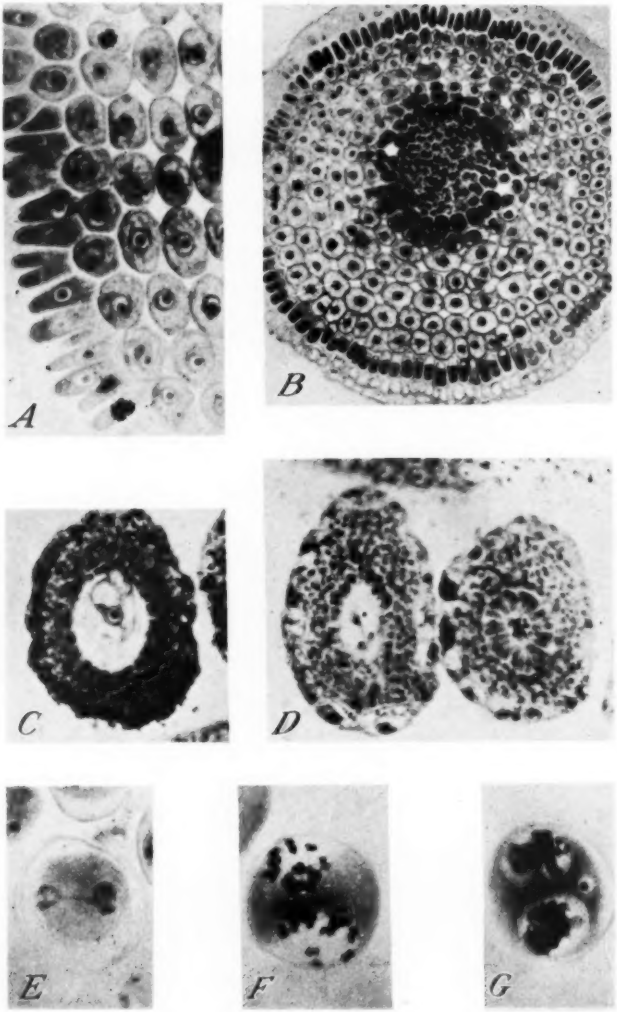
FIGURE 3.—Somatic chromosomes of *Nicotiana glutinosa: A*, The nine identified chromosome types; *B*, haploid plate from haplont 30.180-49; *C*, diploid plate from haplont 30.180-49; *D*, diploid plate from diplont 27.028-6. × 4,750.

are very similar but are easily distinguishable from the chromosomes of the first three types because of their submedian constriction and consequent approach to an L-shape.

The pair of chromosomes composing type 6 is the most easily distinguished of the entire complex. The chromosomes of this type have submedian constrictions, are decidedly L-shaped, and have well-defined proximal satellites. The segments of the pair average 1.40μ and 2.40μ in length.



Nicotiana glutinosa.—Typical metaphase plates from root tips of haplont 30.180–49. *A–F*, Haploid plates. *A* and *B*, Constricted chromosomes. *G* and *H*, Diploid plates. *I*, Typical metaphase plate from root tip of diplont 27.028–6.



Nicotiana glutinosa haplont 30.180–49. *A*, Haploid root tip with a small diploid area in the dermatogen. *B*, Haploid root tip with a large diploid area in the periblem. This chimera nearly surrounds the plerome and greatly distorts the usual symmetrical cell arrangement of the tip. *C*, Embryo sac with a single large nucleus. Compare with *D*. *D*, At left, embryo sac with four characteristic nuclei; at right, characteristic disintegrated embryo sac. *E*, Pollen mother cell. Adnormal interkinesis. Daughter nuclei are connected by chromatin thread. Compare with *F*. *F*, Pollen mother cell. Interkinesis. Chromatin elements are well separated and clearly exhibit homotypic split. This condition is characteristic for the stage in both haplonts and diplonts. This mother cell is larger than that shown in *E* and contains more than 12 chromosome elements; it probably represents an advanced stage of a binucleate prophase (*G*). *G*, Pollen mother cell. Binucleate synizesis, showing characteristic chromatin granule in plasma, and well-separated nuclei.

Upon close examination it is found that the chromosomes of the rod-shaped group may likewise be classified as types (fig. 3). Type 7 comprises the smallest pair of chromosomes of the complex. The chromosomes of this pair average only 3.40μ in length and have practically terminal constrictions. Type 8 likewise comprises a single pair of chromosomes. The constrictions of this pair, however, are further removed from the proximal end than are those of the preceding pair, and the chromosomes are slightly longer, averaging 3.70μ. The chromosomes of the pair composing type 9 are similar to those of type 8 in that they have subterminal constrictions. These chromosomes, however, average 0.80μ longer than those of type 8. Although the chromosomes of the three latter types do not exhibit greater differences than those of types 1, 2, and 3 or types 4 and 5, they are more easily identified. The chromosomes of the rod-shaped group are less twisted and bent than are those of the V-shaped group. Hence, in the former group, differences in form and size are more striking and more easily measured.

A casual observation of haploid plates (pl. 1, *A–F*) clearly shows that there are 9 V-shaped and 3 rod-shaped chromosomes. A detailed study of such plates shows the presence of the 6 types of V-shaped and the 3 types of rod-shaped chromosomes described in connection with the diploid plates. Owing to the fewer number of chromosomes in such plates, the chromosome types are more easily identified than in the diploid plates.

The somatic chromosome types just described, in addition to being characteristic of the two haplonts 30.179-9 and 30.180-49 and all diplonts examined, are also distinguishable in the diploid cells in the root tips of haplonts (pl. 1 and fig. 3). It is likewise possible to distinguish 12 rod-shaped chromosomes and to identify 4 chromosomes of type 6 in one tetraploid metaphase in a diploid root tip from a haplont.

In root-tip metaphase plates of *Nicotiana sylvestris*, the writer (*24*) has noted that the chromosomes often appear to lie in pairs. A similar orientation is apparent in diploid metaphase plates of *N. glutinosa* (fig. 3, *C*). The writer (*25*) has suggested that such orientation is due to similarity of form or the location of spindle-fiber constrictions. This suggestion is supported by the fact that similar chromosomes in haploid *N. glutinosa* plates often appear to lie together or to occupy similar positions within the achromatic figure (pl. 1, *F;* fig. 3, *B*).

OCCURRENCE OF DIPLOID TISSUES IN HAPLONT NICOTIANA GLUTINOSA

Large portions of the root tips of haplont 30.180-49 were entirely or partly diploid (pl. 2, *A* and *B*). Diploid cells and the boundaries between diploid and haploid areas may be rather accurately determined on the basis of cell and nuclear size and chromosome number.

Table 1 shows the number of root tips examined and the approximate age of the plant when they were collected. In the case of each root tip, the data are based on a careful examination from the root cap to the few scattered divisions in the upper zone of cell enlargement.

TABLE 1.—*Frequency of diploidy in root tips of Nicotiana glutinosa haplont 30.180-49* [a]

Age of haplont (weeks)	Number of root tips				Percentage of diploid tips
	Haploid	Diploid	Partly diploid	Total	
16	17			17	0
22	22			22	0
24	33	2		35	5.71
26	40	6	1	47	12.77
28	23	2	1	26	7.69
30	50	[b] 17	1	68	25.00
35	20	9		29	31.03
37	8	10		18	55.56
41	41	30	3	74	40.54
Total	254	76	6	336	22.62

[a] The condition of the haplont at times of root-tip collection is described under Material and Methods. All tips were collected at random.
[b] 1 tip contained a small tetraploid area.

From the data in table 1 it appears that the haplont formed few or no diploid root tips until it was more than 22 weeks old. After this period the percentage of diploid tips gradually increased until the thirty-seventh week, when 55.56 percent of the root tips examined were wholly diploid. As noted in a preceding section, the plant was removed from the field to the greenhouse at the end of 22 weeks, and after 26 and 37 weeks the roots were severely cut back. These facts may account for the sudden appearance of diploid roots at 24 weeks and the slight decrease in the percentage of diploid roots following the twenty-sixth and the thirty-seventh week.

Of the 6 haploid root tips containing $2n$ areas, 2 had diploid areas in the dermatogen (pl. 2, *A*), 2 in the plerome, and 2 in the periblem. Four of the diploid areas were comparatively small and limited to either the zone of cell division or the zone of cell enlargement. One, occurring in the plerome, extended from the zone of cell enlargement to the root cap. The remaining diploid area, occurring in the periblem, extended from the last section examined in the zone of cell enlargement into the root cap, or approximately 820μ. This diploid area (pl. 2, *B*) nearly surrounded the plerome and greatly distorted the usual symmetrical cell arrangement of the root tip. Each of the two last-mentioned diploid areas contained more cells and formed a greater proportion of the root tip in the zone of cell division than in the zone of cell enlargement.

The small tetraploid area in a diploid root tip was situated in the periblem of the upper portion of the zone of cell division. It was composed of from 8 to 10 cells, 1 being in the metaphase stage of mitosis.

Marked roots, which had produced either wholly haploid or wholly diploid tips, were found to continue to produce their respective type for a period of over 2 weeks. Fifteen root tips were examined from the marked haploid root and 19 from the marked diploid root.

Of 19 pieces of rootlets gathered at random from the haplont, 13 had wholly diploid root initials, 5 had wholly haploid initials, and 1 had 1 haploid and 2 diploid initials. There was a total of 32 diploid and 10 haploid initials. This material was collected at the end of 26

weeks. Hence, the high percentage of diploid initials indicates that if the roots had not been cut back at 26 weeks the percentage of $2n$ tips at 28 weeks (table 1) would have been considerably higher.

The callus covering the proximal or root end of the cuttings apparently originated from the vascular cambium. Unfortunately the cells of the forming callus were very irregular in shape and arrangement, so that the mitotic figures were difficult to study. The bulk of the callus, however, was composed of haploid cells. In the forming callus of 1 cutting 4 isolated diploid cells and 1 diploid area composed of approximately 5 cells were visible. In such callus tissues binucleate cells were about as common as diploid cells.

Although several root initials were visible in the callus, in no case did they arise within this tissue. Apparently the initials always originate from the vascular cambium located several sections above the callus. The young initials grow into the callus and finally through it. The callus thus forms a protective tissue for the initials. However, since the initials penetrate the callus more readily than the epidermis, callus tissue must afford less protection than epidermal tissue.

In 1 cutting 5 initials were visible in the callus and 1 in the cortex. In sectioning, the initials were cut at various angles and hence were difficult to study. However, all initials were apparently wholly haploid.

Of the 6 cuttings allowed to develop, only 1 formed diploid root tips in its early history. Two of the first 12 tips collected from this plant were diploid. The first diploid tips on the remaining 5 cuttings appeared approximately 3 weeks later than on the other plant. As in the case of the parent haplont, after the cuttings began to form diploid root tips, the percentage of such tips gradually increased. Of the 130 root tips examined from these cuttings, 22 were wholly diploid and 2 partly diploid.

The significance of the cytological evidence already submitted will be discussed later. It may be noted here that since the extent of diploidy is similar in root tips, marked roots, and root initials, the findings in each confirm those in the others. Moreover, the extent and nature of diploidy in haplont *Nicotiana glutinosa* is very similar to that found in haplonts of *N. tabacum* (*23*), *Crepis capillaris* (*15*), and *Lycopersicum esculentum* (*19*).

CHROMOSOME DOUBLING IN HAPLONT NICOTIANA GLUTINOSA

Several observations indicate that chromosome doubling in the root tips of haplont *Nicotiana glutinosa* occurs through failure of anaphase separation. In the majority of cases the stages of mitoses in haploid tips are as regular as those in diploid tips. Occasionally, however, in haploid figures, the nucleolus does not disappear during the prophase. Remnants or traces of the nucleolus are present during the metaphase and rarely during the anaphase. In the latter stages (fig. 4, *A*), the achromatic figure and the majority of chromosomes are apparently normal. However, some of the chromosomes always appear imperfectly formed and attached to the remnant of the nucleolus. In several of these abnormal figures every chromosome, except those attached to the remnant of the nucleolus, is divided and well separated. It is probable that in some of these abnormal figures,

anaphase separation fails and a single nuclear membrane is formed about the entire diploid chromosome group.

Diploid cells in haploid callus tissues probably originate from binucleate cells (fig. 4, *B*). As previously noted, several binucleate cells were visible in haploid callus, but no evidence was obtained as to their method of formation. The nuclei of these cells always lay in contact with each other or in close proximity. In some cases, probably, the nuclear membrane broke along the lines of contact and the sister nuclei united.

MEIOSES IN POLLEN MOTHER CELLS AND EGG MOTHER CELLS OF HAPLONT NICOTIANA GLUTINOSA [5]

The prophase stages of the egg mother cells and pollen mother cells of haplont *Nicotiana glutinosa* are usually as regular as and very similar to those of diplont *N. glutinosa* or other species of *Nicotiana* (*2*).

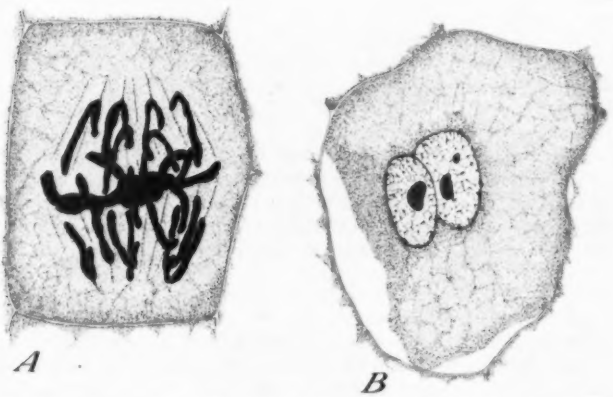


FIGURE 4.—Two cells indicating possible methods of chromosome doubling in *Nicotiana glutinosa* haplont 30.180-49: *A*, Root-tip cell in which a remnant of the nucleolus retards normal anaphase separation; *B*, binucleate callus cell, suggesting union of sister nuclei. × 4,750.

The spireme of the haploid, however, is unpaired throughout the prophase (fig. 5, *A*). Hence the characteristic diploid ring and X-shaped diakinetic pairs are lacking in the haplont (fig. 6, *B*). During diakinesis in the haplont, 12 short, thick, rod-shaped univalents lie about the periphery of the nucleus.

Several twin ovules and binucleate egg mother cells and pollen mother cells are visible (pl. 2, *G*; fig. 5). The sister nuclei of these twin ovules and binucleate cells are always in the same stage of division and are haploid in nature. Binucleate cells usually have a single small chromatin granule in the cytoplasm. Although the origin of this granule is unknown, it is probably connected with that of the binucleate condition.

[5] For convenience, the following symbols used in the University of California publications will be employed to designate the stages of meiosis: I and II, first and second meiotic divisions, respectively; I-M and II-M, first and second metaphase, respectively; I-A and II-A, first and second anaphase, respectively; and I-T and II-T, first and second telophase, respectively.

Since the binucleate cells occur in early meiotic prophase, they probably originate during an archesporial mitosis. Although direct evidence as to their mode of origin is lacking, several conditions indicate that they are not a product of extrusion of the nucleus from one germ cell to another (*13*, *23*). These conditions are, briefly, as follows: (1) Binucleate germ cells are surrounded by an unbroken cell wall; (2) sister nuclei of binucleate pollen mother cells and egg mother cells lie far apart, thus apparently repelling each other; (3) it is extremely difficult, even in degenerate germ tissues, to separate the nucleus from the cytoplasm; and (4) egg mother cells are well separated by somatic tissue and air spaces, yet binucleate egg mother cells occur.

The remaining meiotic behavior is extremely irregular and variable. The 12 rod-shaped univalents contract until they are more or less spherical bodies. Immediately after the disappearance of the nuclear membrane a bipolar spindle is formed. The univalents are scattered over the spindle (pl. 3, *A* and *B*; fig. 6, *D*). They are approximately the same size as the II-M or univalent chromosomes of the diploid species.

One pollen mother cell is visible, with 2 achromatic figures and 12 univalents (fig. 5, *B*) scattered over each spindle. This pollen mother cell is probably an advanced stage of the binucleate prophase condition explained above.

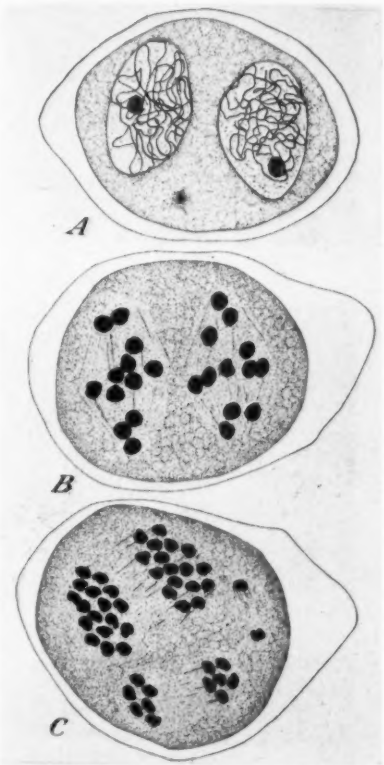


FIGURE 5.—Binucleate pollen mother cells in *Nicotiana glutinosa* haplont 30.180-49: *A*, Pachynema, showing spiremes of nuclei unpaired; *B*, I-M, in which each of the two achromatic figures contains 12 scattered univalents; *C*, II-A, in which the figure presumably arose from a 17 $\begin{cases} >6 \\ >1 \end{cases}$ I-A distribution following spindle fusion. × 2,000.

Since the haplont does not form a I-M equatorial plate there is no foundation for orderly I-A distribution. The univalents are apparently distributed haphazard to the poles (pl. 3, *C*–*E*). All types of chance distribution from 0<>12 to 6<>6 occur. In many cases, however, univalents become laggards and go through an apparent division or fragmentation. This division usually fails and the univalent is finally distributed to a pole, or rarely left in the plasma. Now and then a laggard completes division, and the resulting parts

are likewise included in the daughter nuclei or rarely left in the plasma. Since the products of such division are of various sizes, the division is undoubtedly a fragmentation.

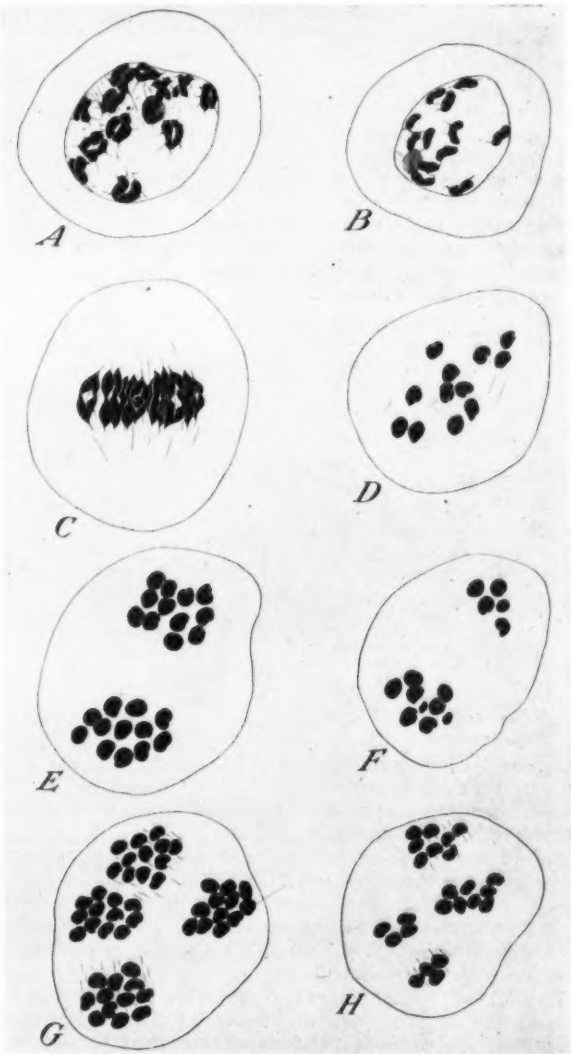
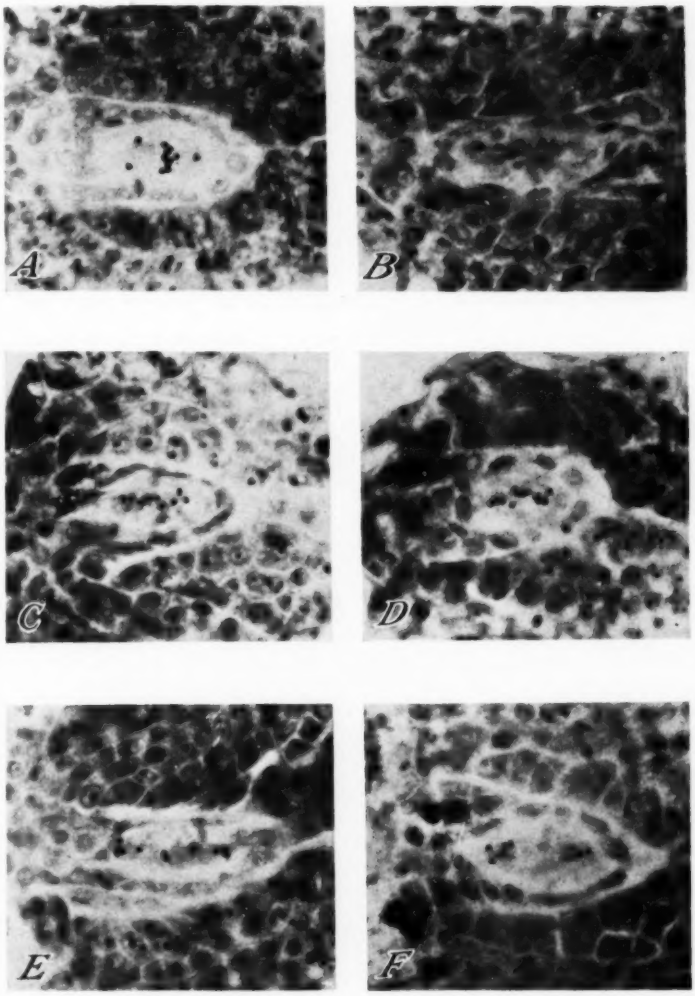


FIGURE 6.—Pollen mother cells of *Nicotiana glutinosa* diplont 30.005-1 (at left) and haplont 30.180-49 (at right): *A* and *B*, diakinesis; *C* and *D*, profile views of I-M; *E* and *F*, polar views of II-M, *F* showing 3 chromosome fragments; *G* and *H*, II-A. × 2,000.

In general, interkinesis is very regular. The chromosomes at this stage are similar to those of the diploid state and exhibit the homo-



Egg mother cells of *Nicotiana glutinosa* haplont 30,180–49. *A*, Profile view of I–M equatorial plate, showing an extremely rare condition. *B*, Profile view of I–M showing a typical figure. *C–E*, Typical I–A figure. *F*, Typical II–M figure.

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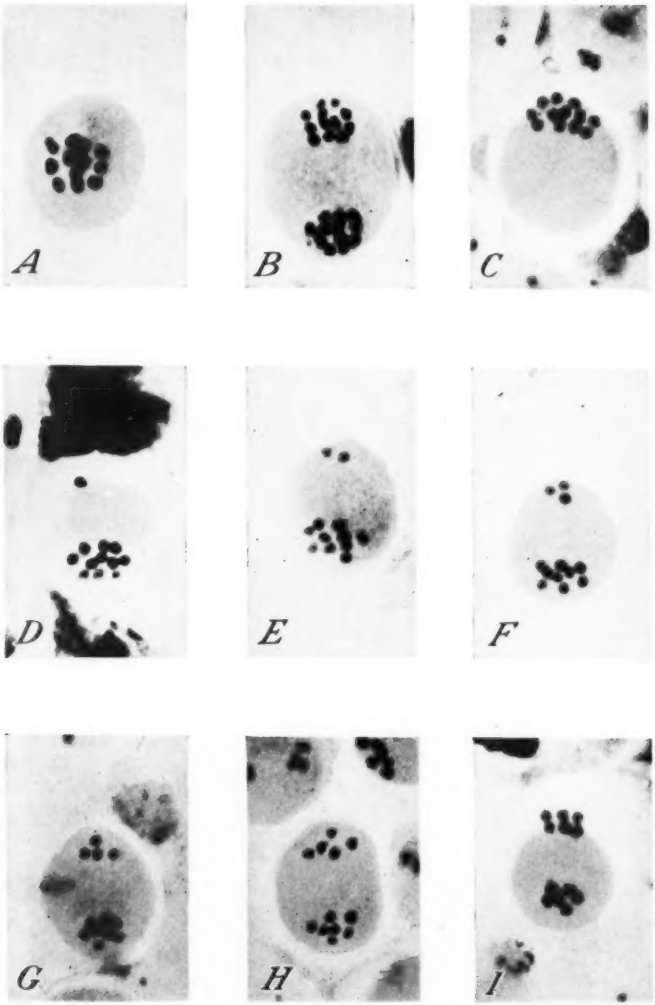

Nicotiana glutinosa. *A* and *B*, Diplont 30.005–1. *A*, Polar view I–M. *B*, Polar view II–M. *C–I*, Haplont 30.180–49; typical II–M figures, resulting from complete 0<>12 to 6<>6 I–A distribution; note relative size of pollen mother cells and chromosome of haplont and diplont.

typic split. The daughter nuclei of one pollen mother cell (pl. 2, *E*) are attached by a fine chromatin thread. This figure probably represents a suspended I-A or semiheterotypic division (*21*). As indicated in table 2, approximately 14.08 percent of the pollen mother cells show chromosomes or chromosome fragments in the plasma.

TABLE 2.—*Chromosomes or fragments in plasma of pollen mother cells at interkinesis*

Number of chromosomes or fragments	Pollen mother cells showing chromosomes or fragments in plasma	
	Number	*Percent*
0	177	85.92
1	19	9.22
2	6	2.91
3	3	1.46
4	1	.49
Total	206	100

As shown in table 3, the II-M plates exhibit various numbers of chromosomes. The table, expanded from 0 to 12, shows a mode of 6, as would be expected from I-A random distribution, and a mean value of 5.56. Typical II-M plates depicting complete random distribution are shown in plate 4, *C–I*, and plate 3, *F*. Although the total number of chromatin units in the plates of each of these pollen mother cells is normally 12, it is occasionally less than this and rarely more. Out of 1,161 pollen mother cells (table 3) in which both plates were countable, 158, or 13.61 percent, showed chromosomes or chromosome fragments in the plasma. This percentage is in agreement with that of such chromatin units found in the plasma during interkinesis. The number of such elements in the plasma ranges from 1 to 4, as in the case of interkinesis. Fifteen, or 1.29 percent, of the 1,161 pollen mother cells (table 3) showed chromosome fragments within the II-M plates. Two of these pollen mother cells showed $3+1<>8+1$ fragments; 2 showed $4+1<>7+1$; 1 showed $4+1<>7+2$ (fig. 6, *F*); 9 showed $5+1<>6+1$; and 1 showed $5<1$ fragment$>6+1$.

It is to be noted (pl. 4, *C*) that II-M plates, which contain all 12 univalents, cannot be mistaken for I-M plates. Such II-M plates are always at the side of the cell or in the approximate position of one of the poles of the I-M spindle. Furthermore, such II-M pollen mother cells are always surrounded by other cells in the same stage of division.

The II-A chromosomes, which are slightly smaller than the II-M chromosomes, are about the same size as those of the diploid at this stage (fig. 6, *G* and *H*). In general behavior II-A is fairly normal. Occasionally, however, laggards occur. These laggards do not show the apparent division exhibited by the laggards in the case of I-A and apparently are ultimately included in one of the granddaughter nuclei. The majority of chromatin units left in the plasma during I are supplied with miniature spindles and divide at II-A. However, occasionally such a unit lacks a II spindle and fails to divide. Since the dividing units are considerably larger than the others, they probably are whole univalents. The chromatin units that fail to

divide and probably the laggards within the major II-A spindles are chromosome fragments.

TABLE 3.—*Number of pollen mother cells showing chromosomes variously distributed on II-M plates*

Type of distribution of chromosomes	Number of pollen mother cells showing chromosomes on—		Total number of pollen mother cells	Type of distribution of chromosomes	Number of pollen mother cells showing chromosomes on—		Total number of pollen mother cells
	Both plates [a]	Single plates [b]			Both plates [a]	Single plates [b]	
0	39	37	115	5	343	312	1,196
12	39	0		7	343	198	
1	48	58	156	6	237	348	822
11	48	2		6	237		
2	60	71	196				
10	60	5		Total	1,003	1,400	3,406
3	88	94	280		1,003		
9	88	10					
4	188	197	641				
8	188	68					

[a] 158 pollen mother cells exhibiting chromosome fragments or chromosomes in the plasma are not included in the table.

[b] Plates exhibiting fragments are not included.

The several counts of pollen mother cells at II-A were 5 of $\overset{7}{\underset{7}{\diamond}} + \overset{5}{\underset{5}{\diamond}}$; 2 of $\overset{6}{\underset{6}{\diamond}} + \overset{6}{\underset{6}{\diamond}}$; 1 each of $\overset{4}{\underset{4}{\diamond}} + \overset{8}{\underset{8}{\diamond}}$ (fig. 6, *H*), $\overset{12}{\underset{12}{\diamond}} + 0$, $\overset{7}{\underset{7}{\diamond}} + \overset{1}{\underset{1}{\diamond}} + \overset{4}{\underset{4}{\diamond}}$, and $\overset{6}{\underset{6}{\diamond}} + \overset{1}{\underset{1}{\diamond}} + 1 + \overset{4}{\underset{4}{\diamond}}$.

The last of these pollen mother cells during I-A probably involved the following conditions: A random distribution of 4<>6; 1 univalent left in the plasma; and the fragmentation of 1 univalent, one part of which was included in the daughter nucleus with 4 univalents and the other part left in the plasma. Two exceptionally large pollen mother cells were visible. One of these (fig. 5, *C*) contained 2 groups of 17 chromosomes, 2 groups of 6 chromosomes, and 2 well-separated chromosomes in the plasma. This unusual pollen mother cell probably represents a more advanced stage of the binucleate condition, which, as previously explained, contains two I-M achromatic figures. The I-A distribution probably was 6<>6 and 11<>1. However, at one side of the pollen mother cell the adjacent spindle poles fused, so that the distribution was $17 \begin{cases} >6 \\ >1 \end{cases}$. Normal homotypic division followed, and the exceptional cell resulted.

As is indicated in table 4, the sporads [6] were highly irregular. Since the spores were equivalent in size in only a few sporads, the segregation in the table is based entirely on the number of spores.

TABLE 4.—*Frequency of occurrence of microspores of various types*

Microspores	Number	Percent
Diads	48	7.52
Triads	18	2.82
Tetrads	456	71.47
Above tetrads	116	18.18
Total	638	

The data in table 4 show that the majority of microsporads were tetrads. Although the spores of these tetrads were in the main unequal in size, a few were equal (fig. 7, *B*). The spores of the latter tetrads were considerably smaller than those of the diads (fig. 7, *D*) or of the tetrads of the diploid species (fig. 7, *A*). Obviously such spores contain less than the haploid chromosome complex and probably are the products of a uniform 6<>6 or similar I-A distribution.

Tetrads, as well as the other types of sporads, frequently contain micronuclei. Figure 7, *D* and *C*, show, respectively, a normal diad and a diad containing two small micronuclei. Approximately 30 to 40 percent of the diads contained such micronuclei. Possibly these diads resulted from a very irregular I-A distribution, such as 1<>11 or 2<>10. If this was the case, then the adjacent products of daughter nuclei lay in such close proximity that they were included in one spore. A few of these micronuclei and the majority of micronuclei of other types of sporads undoubtedly resulted from chromosomes or fragments left in the plasma at I and II.

The spores of normal diads are approximately the same size as those of the tetrads of the diploid state. They probably arise from a 0<>12 I-A distribution and consequently contain a normal haploid chromosome complex.

Triads are possibly produced by a process similar to that by which diads containing micronuclei are produced. The sporads containing more than four spores undoubtedly result from highly irregular meiotic divisions or from binucleate pollen mother cells. Typical sporad types are shown in figure 7.

Up to II-T the behavior of the egg mother cells and pollen mother cells is practically identical. Possibly, however, the behavior of the egg mother cells is slightly more uniform and the mean I-A distribution nearer the mode. However, following II-T, the majority of egg mother cells rapidly disintegrate to a deep-staining mass. As degeneration proceeds, the surrounding somatic cells elongate and gradually supplant the egg mother cells (pl. 2, *D*). After such replacement the somatic cells fail to develop further and finally the entire ovule disintegrates.

[6] Although in the majority of plants the meiotic divisions produce a group of 4 spores, in numerous cases the divisions are highly irregular and from 1 to 12 or more spores are produced. Cases are known where 2 spores are characteristically formed. In literature dealing with these conditions such a statement as "the majority of tetrads contain only four spores" is rather common. It therefore appears that the term "tetrad" (*tetra* (Greek), four; and *ad* (Latin), toward) or "tetrad stage" is not expressive of the condition that obtains. The term "sporad" (*sporo* (Greek), seed, spore) is proposed for this stage or group of cells. Alternative terms, such as "sporcorymbus" (*corymbus* (Greek), cluster, group) are not in keeping with the terms "tetrad", "diad", etc., and are more cumbersome.

Although an occasional egg mother cell develops beyond II-T, such development is never normal and is rarely duplicated in another egg mother cell. The megasporads which develop always have at least two spores, the nuclei of which are approximately the same size as those of the microdiad. In addition to the large spores, there are occasionally one or two small ones. No definite evidence was obtained as to which spore develops into the megaspore and finally produces the female gametophyte. However, judging from the size of

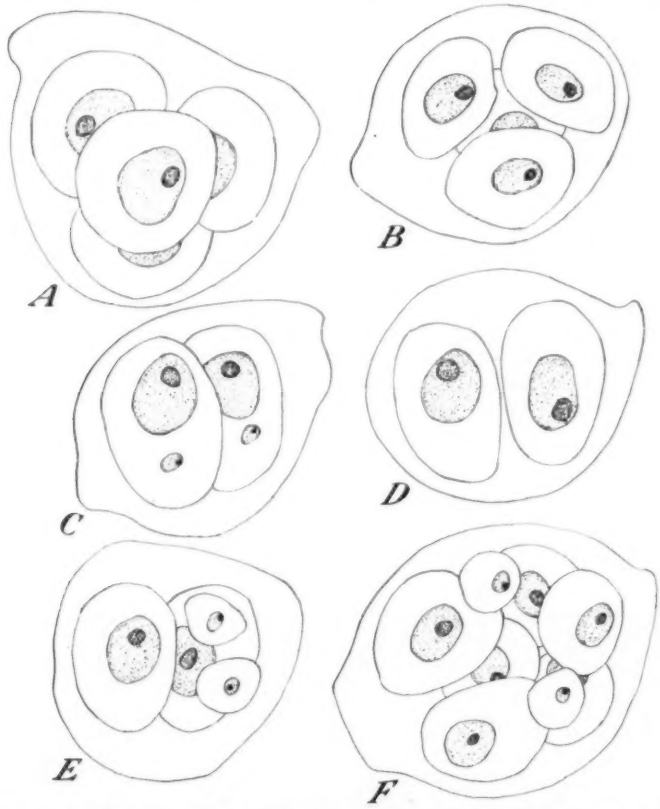

FIGURE 7.—Comparative microsporads of diploint (30.005-1) and haploint (30.180-49) *Nicotiana glutinosa*: *A*, Diploint tetrad; *B–F*, representative haploint sporads. × 2,000.

gametophytic nuclei and from a few questionable chromosome counts of these nuclei, only spores containing approximately 12 chromosomes develop.

Further development is highly irregular and no definite stages occur. In the majority of cases, apparently, rapid division follows and from 4 to 7 nuclei are formed (pl. 2, *D*). Although in a few cases the nuclei are scattered throughout the gametophyte, no definite arrangement occurs. In the majority of cases the nuclei are in a single

compact group. Whether these nuclei remain in this condition until degeneration or whether they fuse is undetermined. However, later in development, the appearance of extremely large nuclei (pl. 2, *C*) indicates that the latter condition occurs. Such nuclei are visible in ovary material collected during anthesis. Although several of these large nuclei are in prophase, no gametophyte containing more than one such nucleus is visible. Counts of the latter nuclei indicate that they contain 35 or more chromosomes. Approximately at the time when fecundation should take place, degeneration starts, and all ovules, including those in which somatic cells have replaced the embryo sac, rapidly disintegrate.

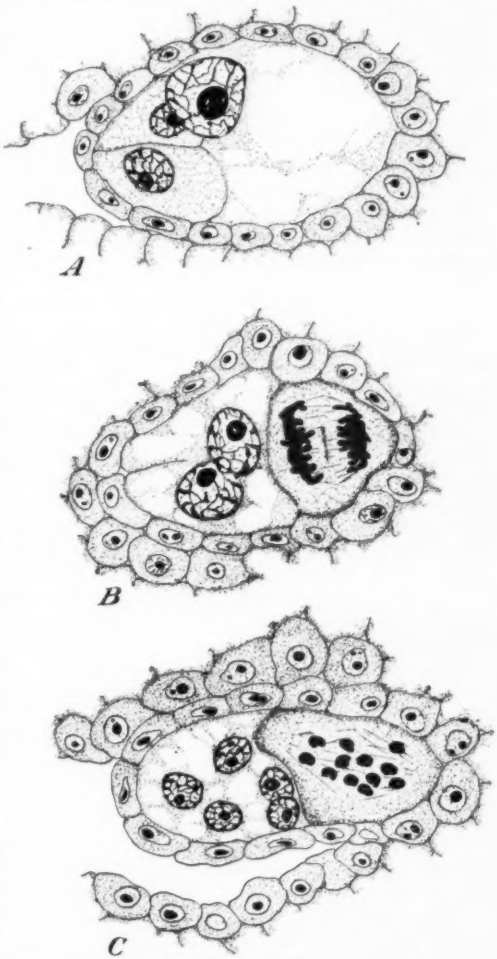

FIGURE 8.—*A–C*, representative female gametophytes of *Nicotiana glutinosa* haplont 30.180-49. × 950.

As stated previously, no definite arrangement of stages occurs, hence no mature or normal embryo sac is formed. The most nearly normal condition is shown in figure 8, *A*. The three nuclei of this figure may represent the two synergids and the egg. In this case, the egg is extremely large in proportion to the synergids and may represent the union of several nuclei. Figure 8, *B* and *C*, shows two of the many irregularities. Although no explanation is offered for these unusual figures, apparently in the one shown in figure 8, *B* and *A*, a polyploid somatic cell is replacing the embryo sac. Figure 8, *C*, represents a condition among well-advanced ovules and hence should be beyond the meiotic divisions. The nuclei at the bottom of the embryo sac likewise indicate an advanced stage. However, the 12 chromosomes

in the upper part of the embryo sac are the size of univalents and have a decidedly meiotic form.

Since preparations of the male gametophyte are extremely difficult to obtain, no study of its development was made. However, the young pollen grains are extremely variable in size and shape, and only a few appear equivalent to those of the diploid species. During anthesis a few grains of the latter species were found in a whitish powder obtained from the anthers.

DISCUSSION

Inasmuch as the meiotic behavior of the *Nicotiana glutinosa* haplont is similar to that of other haplonts, especially in *Nicotiana*, this discussion will be confined mainly to somatic behavior and phenomena concerning such behavior. Moreover, since the literature dealing with haplonts has recently been reviewed by Gates and Goodwin (*8*) and by Kuhn (*18*), it will not be summarized here.

CHROMOSOME NUMBER AND MORPHOLOGY

That the normal somatic chromosome number is 24 (*3*) is confirmed by the present study of the chromosomes in diplont and haplont *Nicotiana glutinosa*. Since no pairing occurs during meiosis in haplont *N. glutinosa*, it is reasonably certain that 12 is the basic haploid number of chromosomes for the species.

Somatic chromosomes of *Nicotiana glutinosa* are similar in shape to those of *N. tabacum*, in which $n=24$ (*23*); to those of *N. alata*, in which $n=9$ (*1, 22*); and to those of *N. sylvestris*, in which $n=12$ (*24*). They are, however, considerably smaller in diameter than the chromosomes of these species and are not so variable in size. The chromosomes of *N. glutinosa* differ from those of *N. longiflora*, in which $n=10$ (*14*), in that the chromosomes of the latter species are uniformly rod-shaped. To some extent, therefore, specific chromosome distinctions separate the complex of *N. glutinosa* from the complexes of the other species mentioned.

According to the data and figures presented by the authors just cited, the chromosomes of *Nicotiana sylvestris* and *N. tabacum* are more nearly alike than those of any other of the species mentioned. In interspecific hybrids it has been shown that chromosome affinity is much greater in the *N. tabacum*-*N. sylvestris* combination than in any other combination of these five species [7] (*3; 4, p. 181; 7, p. 273–274; 9; 12*).

COMPARISON OF DIPLOIDY IN HAPLONT ROOTS AND TETRAPLOIDY IN DIPLOID ROOTS

As stated previously, the extent of diploidy is similar in root tips, marked roots, and root initials of haplont *Nicotiana glutinosa*. Only 2.21 percent of the haploid root tips gathered at random were partly diploid. This percentage indicates that the occurrence of diploidy in haplont roots is less frequent than is commonly believed (*14, 17, 19, 23*). In fact, diploidy in haplont roots does not appear to originate more frequently than does tetraploidy in diploid roots. Of the wholly diploid root tips of haplont *N. glutinosa*, 1.02 percent showed

[7] The degree of affinity between *Nicotiana alata* and *N. longiflora* seems to be questionable (*7*).

tetraploid chimeras. In a previous paper the writer (*24*) has shown that in diploid *N. sylvestris*, 2.50 percent of the root tips contain tetraploid chimeras, and he has reported a tetraploid chimera in *N. sanderae* Sander. Since 1930 the writer has observed such chimeras in *N. rusbyi* Britt. and *N. trigonophylla* Dun. Although in the last three cases no records were kept, it is certain that in each case tetraploidy originated as frequently as diploidy in haplont *N. glutinosa* root tips.

On the other hand, unless some phenomenon aside from frequent origin occurs, the high percentage (55.56) of wholly diploid root tips cannot be explained. The writer (*24, p. 360*) has stated:

> The diploid cells of the haplonts are by far the most vigorous cells of the entire root tip and multiplication is more rapid in the diploid cells of such a root. The descendants of a diploid root initial in a haploid plant will in all probability persist in that meristem, and possibly in time dominate the tissues of the organ.

Such a change is precisely what happened in the *Nicotiana glutinosa* haplont 30.180-49. It has already been shown that the two large diploid chimeras in haploid root tips contain more $2n$ cells in the zone of cell division than in the zone of cell enlargement. It is also shown that the percentage of wholly diploid root tips increased from 0 to 55.56.

Such development and domination of diploidy in roots of haplont *Nicotiana glutinosa* is in direct contrast to that found in the case of tetraploidy in roots of diplont *N. sylvestris*. In the latter case the writer (*24, p. 360*) has stated:

> That the larger sector * * * is gradually reaching its maximum size is indicated by the comparatively uniform decrease in the number of tetraploid cells from the region of elongation to the growing point. * * * If the root tip had been allowed to grow, this sector, and probably the majority of other sectors, would have become "islands" of tetraploid cells.

Hence, the size of $2n$ and $4n$ areas which develop from such initial cells in the roots of haplonts and diplonts is apparently determined by growth-rate relations of these tissues. Consequently such growth-rate distinctions must also determine the frequency of wholly $2n$ and $4n$ roots, since these roots must arise from such areas in the pericycle.

COMPARISON OF ROOT AND SHOOT IN RELATION TO POLYPLOIDY

It is well known that diploidy in haplonts occurs more frequently in roots than in shoots (*23, 15, 19*). A similar condition exists in the case of tetraploidy in diplonts. Although the reason for the greater prevalence of these chimeral tissues in the root is apparently unknown, several clues are afforded by the nature of the root and shoot, especially by the relative amount of meristematic tissue in these organs and the relative degree of protection such tissues receive.

Undoubtedly the number of apical meristems in the root system is greater than in the shoot. Meristematic tissue in the shoot is covered with a more effective protective tissue than is that in the root. Moreover, the apical meristem of the shoot receives additional protection from young leaves and is therefore better shielded from environmental changes that may initiate polyploidy. Hence, it seems probable that polyploidy originates more frequently in the root than in the shoot.

Although the environmental variations to which the shoot is subjected are ordinarily greater than those that affect the root, as great

or greater variations are usually encountered by roots grown for cytological investigations. Such roots, generally from potted plants that soon become more or less pot-bound, are subjected to various and sudden changes in temperature and moisture. Since the root is not so effectively constructed as the shoot to endure such variations, the greater frequency of polyploidy in roots grown under such conditions is not surprising.

The behavior of haplont *Nicotiana glutinosa* is in harmony with the foregoing facts. It was shown in a preceding section that all roots gathered from field plants or from plants (cuttings) grown in an ample volume of soil were wholly haploid. On the other hand, as soon as the plants become more or less pot-bound, diploidy suddenly occurred.

It is probable, however, that tetraploid and also diploid chimeras are of more frequent occurrence in shoots than has been observed, since apical meristems of shoots are very seldom examined.

HAPLONT CALLUS TISSUE IN RELATION TO DIPLOIDY

As previously shown, the callus at the proximal ends of shoot cuttings of haplont *Nicotiana glutinosa* originates from the vascular cambium and is composed chiefly of haploid cells, although a few diploid and binucleate cells occur. The mode of origin is in agreement with that reported by Priestley and Swingle (*20*). The occurrence of diploid and binucleate cells in the proximal callus is probably due to the effect of environmental changes on the unprotected dividing cells. Such diploid cells have no effect on the composition of roots produced by cuttings, since new roots originate from the vascular cambium several sections above the callus.

No distal callus is formed in haplont cuttings of *Nicotiana glutinosa*. However, distal callus similar in composition to the proximal callus of haplont cuttings of *N. glutinosa* has been reported by Jørgensen (*16*) and by Lindstrom and Koos (*19*). The occurrence of diploid cells in the callus of decapitated haplonts may be attributed to causes similar to those resulting in diploid cells in proximal callus. However, unlike the diploid cells of the proximal callus, those of the distal callus may have a profound effect on the composition of new tissues Since Priestley and Swingle (*20*) found that buds are exogenous in origin, whereas roots are endogenous, the occasional occurrence of diploid and tetraploid shoots from decapitated haplonts may be accounted for by the chimeral composition of the distal callus.

MEIOTIC FEATURES OF HAPLONT NICOTIANA GLUTINOSA

As previously stated, the meiotic behavior of haplont *Nicotiana glutinosa* is very similar to that of other *Nicotiana* haplonts (*2*, *5*, *6*, *11*, *17*, *23*). Because of this similarity and since Hollingshead (*14*) has recently discussed the meiotic resemblances and differences of haplonts, it appears unnecessary to present any further compilations here. Hence, the new, unusual, and important meiotic and gametophytic features of the *N. glutinosa* haplont are merely enumerated. These are as follows:

The occurrence of binucleate egg mother cells and pollen mother cells and of twin ovules during the meiotic prophase.

The complete lack of any observable conjugation during meiosis.

The lack of the homotypic split or apparent equational division of univalents during I.

The apparent fragmentation of univalents during I-A.

The formation of two I-M achromatic figures in binucleate germ cells.

The success of I-A random distribution, the 12 univalents being distributed, in nearly all cases, in all types of distribution from 0<>12 to 6<>6.

The very rare occurrence of semiheterotypic divisions.

The II-M mode of 6 and mean value of 5.56.

The occurrence of chromosome fragments at II-M.

The fairly normal II-A distribution.

The occurrence of II-A pollen mother cells containing 48 chromosomes, which probably represent advanced stages of the binucleate condition. The possible fusion of the I-M achromatic figures in binucleate cells, giving rise to II-M and II-A plates containing more than 12 chromosomes.

The occurrence of highly abnormal sporads and occasional diads, the spores of which contain the complete haploid chromosome complex.

The disintegration of the majority of egg mother cells following II-T, and their gradual replacement by somatic cells.

The formation of an occasional female gametophyte.

The great irregularity and lack of definite stages in the female gametophyte.

The apparent fusion of nuclei in the female gametophyte and the occurrence of polyploid nuclei.

The degeneration of all ovules at the approximate time when fecundation should occur.

The occurrence of a few apparently normal pollen grains.

SUMMARY

Two additional haplonts of *Nicotiana glutinosa* were found during the spring of 1930 in the botanical gardens of the University of California. One of these plants is described and figured.

The somatic chromosome complex of *N. glutinosa* consists of 24 chromosomes. The basic haploid number is apparently 12.

The chromosomes of the somatic garniture exhibit morphological differences permitting the recognition of nine distinct types.

Diploidy frequently occurs in the root system of haplont *N. glutinosa*. The percentage of wholly diploid root tips observed increased from 0 at 22 weeks to 55.56 at 37 weeks. Diploid areas were present in 2.21 percent of the haploid root tips, and 1.02 percent of the diploid root tips contained tetraploid areas.

Diploid areas extending from the zone of cell division into the zone of cell enlargement contain more cells in the former zone and hence are larger than in the latter zone.

Marked diploid and haploid roots produced wholly diploid and wholly haploid tips, respectively.

Of 19 pieces of rootlets, 13 had wholly diploid root initials; 5 had wholly haploid initials; and 1 had 1 haploid and 2 diploid initials.

The basal callus tissue of cuttings is composed mainly of haploid cells, although a few diploid and binucleate cells occur.

Root initials in the callus originate several sections above the callus. All initials are wholly haploid.

Out of 6 cuttings, only 1 had diploid root tips in its early history.

Chromosome doubling in root tips is possibly due to the failure of anaphase separation, following the retention of the nucleolus to this stage. Chromosome doubling in callus tissue is possibly due to the fusion of the nuclei of binucleate cells.

Meiotic and gametophytic behavior is exceptionally irregular, and several new and unusual features occur. The term "sporad" is suggested for the spore or group of spores (tetrads, triads, microcytes,

etc.) resulting from meiotic divisions. A summary of meiosis is given in the final section of the discussion.

Evidence is presented that the high percentage of diploidy in haplont roots of *N. glutinosa* is due to the relative growth relations of $2n$ and n tissues rather than to the frequent origin of $2n$ initials.

Growth rates of $4n$ and $2n$ tissues in relation to the occurrence of tetraploid chimeras in diplonts are discussed.

Evidence is presented that diploidy in haplont roots is probably due to the effect of frequent and sudden environmental changes on poorly protected meristematic tissue.

Evidence is presented that the basal-callus tissue of cuttings forms a protective coat for adventitious root initials. Comparisons are drawn between the basal callus and root initials, and the conditions presumably existing in the apical callus and bud initials.

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LIVER-SPOT DISEASE OF PECAN FOLIAGE CAUSED BY GNOMONIA CARYAE PECANAE, NOV. VAR.[1]

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INTRODUCTION

In the vicinity of Shreveport, La., during the early summer, a leaf spot may be found on the foliage of the pecan (*Hicoria pecan* Brit.). This type of leaf spot was first reported on the pecan by Demaree,[3] who found it in Louisiana and Texas and called it "western sooty spot." The color of the spot is first dresden [4] and later cinnamon brown, but never black or sooty. It is therefore suggested that the name be changed to "liver spot" as being more descriptive of the disease.

DISTRIBUTION AND ECONOMIC IMPORTANCE

The liver-spot disease has been under observation by the writer for several years, and during the season of 1931 an extensive survey was made to determine its distribution and its economic importance to the pecan industry. At that time the disease was prevalent in central Mississippi, Arkansas, northern Louisiana, and Texas. Unlike most other parasitic diseases of the pecan, it seems not to thrive in regions of high humidity, especially near the Gulf coast of Louisiana and Mississippi, and it was not found in southern Alabama, southern Georgia, or northern Florida during July 1931 when some orchards of these States were visited by the writer.

Liver spot is not considered to be as serious as some other parasitic diseases of pecans, especially scab caused by *Cladosporium effusum* (Wint.) Demaree, but it is severely damaging to pecan foliage in many localities in the southwestern part of the United States. Since it is sometimes accompanied by other leaf spots, especially downy spot caused by *Mycosphaerella caryigena* Demaree and Cole, and vein spot caused by *Leptothyrium nervisedum* Cole, it is difficult to estimate accurately the damage due to this disease.

The fungus attacks trees of low vitality, particularly those thickly crowded or growing in soil low in humus; it also attacks vigorous trees growing in the most fertile soil of the Red River Valley. During the early fall of 1930 and 1931 some of the leading varieties of pecan in western Louisiana and eastern Texas were prematurely defoliated by the liver-spot pathogene.

DESCRIPTION OF THE DISEASE

The disease first appears on the lower surface of the leaflets, usually along the midrib (fig. 1), as umber or dresden-brown circular dots,

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[2] The writer acknowledges his indebtedness to C. L. Shear, Division of Mycology and Disease Survey, for assistance in the classification of the causal organism; to John W. Roberts and J. B. Demaree, Division of Fruit and Vegetable Crops and Diseases, for criticism of the manuscript; and to Edith K. Cash, Division of Mycology and Disease Survey, for assistance in translating the technical description into Latin.

[3] DEMAREE, J. B. DISEASES OF PECANS IN THE SOUTHERN STATES. U.S.Dept.Agr. Farmers' Bul. 1672, 28 pp., illus. 1931.

[4] RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 pp., illus. Washington, D.C. 1912.

resembling the color of liver. These dots enlarge and may reach a diameter of 1 cm, rarely more, except where they coalesce. The color remains the same until late summer or early fall, when it changes gradually from dresden to cinnamon brown and pycnidia appear in the central parts of the diseased areas (fig. 2). At this time the diseased areas become visible on the upper surface of the leaflets. Figure 3 shows a leaflet with about two thirds of its surface discolored by the fungus and the lower edge darkened by the masses or groups of pycnidia. At this stage of the disease the leaflets usually are shed.

FIGURE 1.—Pecan leaf showing early stages of liver spot.

THE CAUSAL FUNGUS

CONIDIAL STAGE

During late summer, when the diseased areas change from dresden to cinnamon brown, groups of reddish-brown pimplelike pycnidia form in the center of the spots. The production of pycnidia may continue until the entire spot is covered. They are at first subcuticular, later superficial. The pycnidia are not firmly embedded in the host tissue, as the outer covering begins at the top of the epidermal cells. The pycnidial covering consists of very small, irregularly shaped cells, which have thin walls that are difficult to distinguish clearly without the aid of high magnification. This covering varies in thickness from 2 to 3 cells at the base to 4 to 6 cells at the top. There is no ostiole. The base of the pycnidium is made up of stromatic layers, 2 to 4 cells thick, which apparently dissolve the epidermis. From these stromatic cells hyphae extend into the spongy parenchyma of the host (fig. 4, *B*). The conidia are borne at the apical end of short conidi-

ophores, which are slightly longer than the conidia. The conidiophores are simple, hyaline, and 1 to 2 septate. The conidia are continuous, hyaline, curved, fusoid, large at the basal end, and attenuated toward the apex.

FIGURE 2.—Pecan leaf showing both early and late stages of liver spot. Fruiting bodies are appearing in the lighter areas.

PERITHECIAL STAGE

In the vicinity of Shreveport, La., perithecia begin to develop in late winter and reach maturity in the early spring. They develop mostly on the lower surface of the leaves, among the old pycnidia, most often singly, but sometimes in groups of twos or threes. The perithecia, which are embedded deep in the host tissues, extend through the spongy parenchyma and penetrate the palisade cells (fig. 4, *E*). The long beaks of the perithecia, which have well-developed ostioles, extend above the leaf and may be seen with the unaided eye. The perithecia are somewhat flask-shaped and are composed of thick-walled cells. The perithecial walls are dark brown, 2 cells thick at the bottom and top, and 3 to 4 cells thick on the sides.

The asci are hyaline, eight-spored, and cylindrical; they are not accompanied by paraphyses. At the apical end they have a pore, which is characteristic of the genus *Gnomonia*. The ascospores are straight or curved, hyaline, guttulate, and one-septate, both cells being about equal in size, with a slight constriction at the septum. The spores are enveloped in a gelatinous substance without defined cell walls and with pointed hyaline appendages at each end, the combined length of two appendages being about equal to the length of the spore. Before the spores are discharged from the ascus, the appendages lie close alongside the spores (fig. 4, *D*), extending after the discharge of the spores.

IDENTITY OF THE FUNGUS

Ellis and Everhart [5] described the imperfect stage of a fungus on hickory leaves in 1891 as *Gloeosporium caryae*. Ellis and Everhart [6] re-

[5] ELLIS, J. B., and EVERHART, B. M. NEW SPECIES OF FUNGI FROM VARIOUS LOCALITIES. GLOEOSPORIUM CARYAE ELL. AND DEARNESS. Acad. Nat. Sci., Phila. Proc. 1891: 82. 1892.

[6] ELLIS, J. B., and EVERHART, B. M. THE NORTH AMERICAN PYRENOMYCETES, A CONTRIBUTION TO MYCOLOGIC BOTANY, p. 326, Newfield, N.J. 1892.

FIGURE 3.—Pecan leaf showing advanced stage of liver spot. Midrib and veinlets are dying; fruiting bodies are abundant. Leaves are usually shed soon after reaching this stage.

ported *Gnomonia setacea* (Pers.) Ces. and De Not., var. *macrospora* Ell. and Ev., as occurring on fallen leaves of *Quercus*, *Castanea*, and *Carya* (*Hicoria*) near Newfield, N.J. Matz[7] reported a two-spored *Gnomonia* on living pecan leaves in Florida in 1917. Wolf[8] collected, in the vicinity of Auburn, Ala., an imperfect fungus on hickory leaves, which he called *Gloeosporium caryae* Ell. and Dearn., and by placing the leaves outdoors over winter, he found the perfect stage the following spring. This perfect stage he described as *Gnomonia caryae*. So far as the writer has been able to determine, Demaree[9] was the first to collect the imperfect stage of the liver-spot fungus on pecan leaves. He considered the causal organism to be *Gloeosporium caryae* var. *curvisporum* Dearn.

In the early studies of the liver-spot pathogene, the general morphological characteristics of the fungus indicated that it should be classified as belonging to the genus *Gloeosporium*. Since *Gloeosporium caryae* is common on hickory leaves in Louisiana and Mississippi, collections of hickory leaves bearing the fun-

[7] MATZ, J. AN UNDESCRIBED GNOMONIA ON PECAN LEAVES. Fla. Agr. Expt. Sta. Ann. Rpt. 1917: 89R–94, illus. 1918.

[8] WOLF, F. A. A NEW GNOMONIA ON HICKORY LEAVES. Ann. Mycol. 10: 488–491, illus. 1912.

[9] DEMAREE, J. B. See footnote 3.

gus were made in different parts of these States, and type specimens of *G. caryae* were secured from the herbarium in Washington, D.C. Microtome sections of the hickory and the pecan leaves showed that the fruiting bodies of what has been called *G. caryae* on hickory leaves and the imperfect stage of the liver-spot fungus on pecan are not acervuli but are pycnidia (figs. 4–7). The cell structures in the covering of the pycnidia are quite distinct under high magnification. C. L. Shear, of the

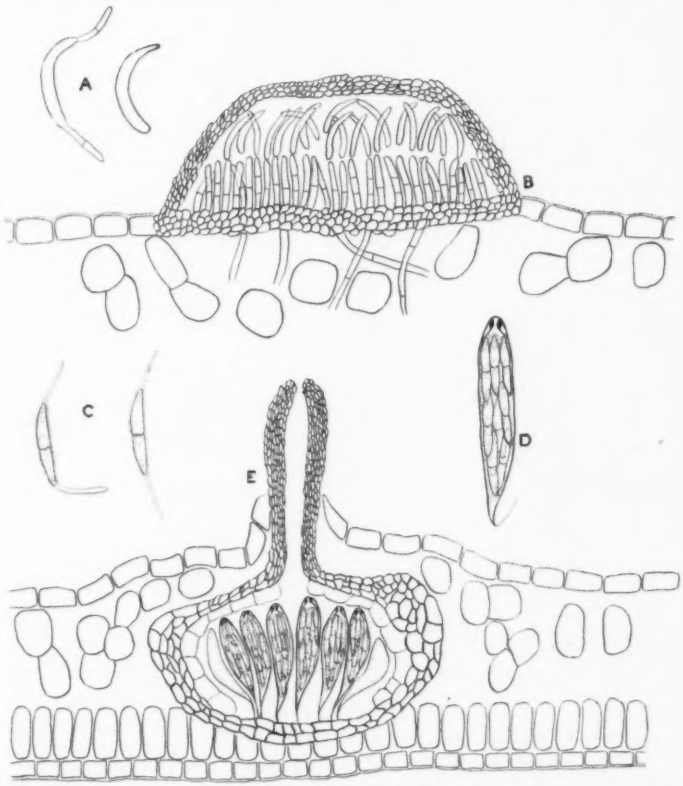

FIGURE 4.—The liver-spot fungus on pecan: *A*, Types of conidia and germination (×1,800); *B*, pycnidium (× 900); *C*, types of ascospores and germination (× 1,200); *D*, ascus with eight ascospores (× 900); *E*, perithecium embedded in leaf (× 600).

Bureau of Plant Industry, United States Department of Agriculture, examined the microtome sections and suggested that the imperfect stages belong in the genus *Leptothyrium* and not *Gloeosporium*. A comparison of the imperfect fungus on pecan and that on hickory leaves showed that the pycnidia of the liver-spot fungus on pecan commonly appear in groups, whereas on the hickory they are usually scattered over the entire leaf. The pycnidial covering of the liver-spot fungus is 2 to 6 cells thick, whereas the covering on the hickory fungus is only 1 to 2 cells thick. The conidia of the liver-spot fungus are long and slender (15μ to 20μ long and 2μ to 3.5μ wide); those of the hick-

ory fungus are shorter (7μ to 10μ long and 2μ to 3.5μ wide). Wolf reported the latter conidia to be 7μ to 10μ long and 1.5μ to 2μ wide.

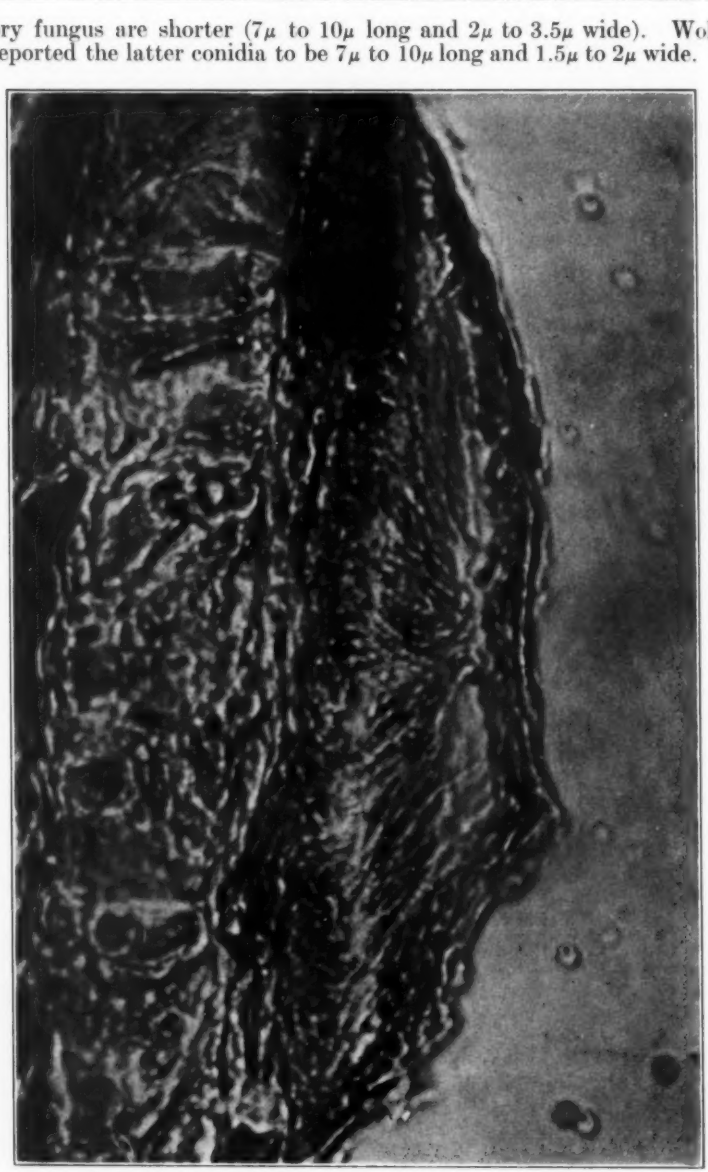

FIGURE 5.—Photomicrograph showing conidial stage of the liver-spot fungus on pecan. The pycnidial covering is composed of several layers of cells (× 1,400).

In making cross-inoculations with the conidia from pecan or from hickory, the writer was never able to get as high a percentage of

infection by inoculating hickory with the conidia from pecan, or vice versa, as by inoculating either species with conidia from the corresponding host.

Type specimens of *Gloeosporium caryae* var. *curvisporum* when examined appeared distinctly different, both macroscopically and microscopically, from the imperfect forms of liver spot and the fungus

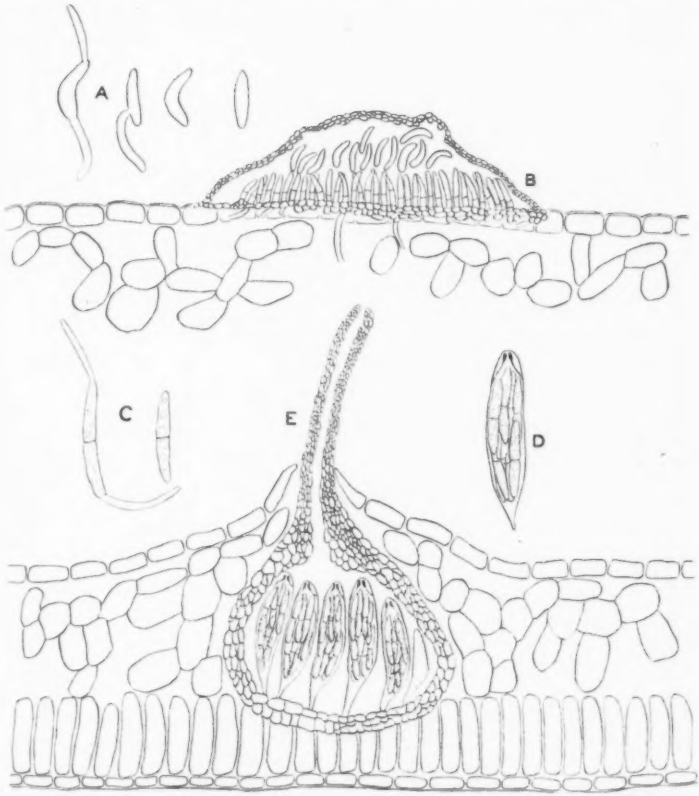

FIGURE 6.—*Gnomonia caryae* Wolf on hickory: *A*, Types of conidia and germination (× 1,800); *B*, pycnidium (× 900); *C*, types of ascospores and germination (× 1,200); *D*, ascus with eight ascospores (× 900); *E*, perithecium embedded in leaf (× 600).

on hickory. They conform more to the *Gloeosporium* than to the *Leptothyrium* type.

Because of the fact that there is a morphological similarity between the imperfect stages of the liver-spot fungus and the hickory fungus, and because the inability to get a high percentage of infection by cross-inoculating the fungi may have been due to physiologic strains rather than separate species, the writer suggests that the imperfect fungus on hickory be called *Leptothyrium caryae* and that on pecan *L. caryae* var. *pecanae*. Both *L. caryae* and *L. caryae* var. *pecanae* are different from *L. nervisedum* which occurs on the foliage of pecan.

FIGURE 7.—Photomicrograph showing conidial stage of *Gnomonia caryae* Wolf on hickory. The pycnidial covering is composed of one or two layers of cells (× 1,400).

The last-named fungus confines its attacks to the vascular system of the leaves, and it differs in morphological characters from the other two fungi. Its pycnidial covering is dark brown to black, its cells are indefinite, and its conidia are oblong to ovate. In cultural characters also it differs from the other pathogenes.

Pecan leaves bearing pycnidia of the liver-spot fungus were placed in cages outdoors to overwinter in the vicinity of Shreveport, La. Examinations in the early spring disclosed mature perithecia among the old pycnidia. Because of the morphological characteristics of the perithecia, the fungus was placed in the genus *Gnomonia*. The spores of this fungus have appendages and, since Ellis and Everhart [10] reported a *Gnomonia* on *Carya* (*Hicoria*) having spores with appendages, a comparison of the two fungi was made.

An examination of type specimens of *Gnomonia setacea* (Pers.) Ces. and DeNot. and *G. setacea* var. *macrospora* disclosed the fact that the ascospores either were immature or had deteriorated so much that a fair comparison could not be made. The former is found on pecans and oaks in the South and the latter on hickory in the East, especially in New Jersey. The perithecia of these organisms are not confined to any part of the leaflets, but are scattered over the entire leaf surfaces. There is no evidence of old pycnidia among the perithecia, nor has there been a conidial stage connected with either organism, so far as the writer has been able to ascertain. The descriptions of *G. setacea* and *G. setacea* var. *macrospora*, according to Ellis and Everhart,[10] indicate that these forms are distinct from *G. caryae* Wolf on hickory, being 4- to 8-spored and septate in the middle without constriction. During the studies of *G. caryae* and the liver-spot fungus, the writer has never observed an ascus with less than eight spores, and the spores also show a slight constriction at the septum (figs. 4 and 6). Furthermore, the spores of both of these forms are larger than those of either *G. setacea* or *G. setacea* var. *macrospora*. Matz [11] also described a *Gnomonia* on pecan, but since that fungus produces its fruiting bodies on living leaves and since two spores, rarely three or more, are produced in an ascus, it is considered a species distinct from both *G. caryae* and the liver-spot fungus.

The morphological characteristics of Wolf's *Gnomonia* on hickory and those of the fungus on pecan, herein described, are similar, except that the spores of the latter are usually accompanied by appendages composed, perhaps, of a gelatinous substance without a defined cell wall. Appendages were not reported on the spores of *G. caryae* by Wolf, nor were they found by the writer (fig. 6, *C*). The spores of *G. caryae* are longer than those of the liver-spot fungus; the former are 25μ to 33μ long and 5μ wide, whereas the latter are 22μ to 28μ long by 3μ to 4μ wide. The measurements of the pecan spores do not include the appendages.

After a number of unsuccessful attempts to get infection with ascospores from *Gnomonia caryae* and the liver-spot fungus, inoculations were made with conidia from the ascospore cultures. As previously stated, a higher percentage of infection was obtained when the inoculum was placed on the host species (hickory and pecan, respectively) than when cross-inoculations were made.

[10] Ellis, J. B., and Everhart, B. M. (See footnote 5.)
[11] Matz, J. (See footnote 7.)

The growth characters of the two fungi on artificial media were very much alike. However, conidia were produced in pycnidia when cultured from either the imperfect or the perfect stage. Wolf[12] found that when cultures were made from the conidial stages of *Gnomonia caryae* the conidia were not borne in acervuli but on hyphae or short conidiophores. The writer also found this to be true.

Because of the similarity between *Gnomonia caryae* and the liver-spot fungus, the writer suggests that the latter be designated *Gnomonia caryae* var. *pecanae*.

TECHNICAL DESCRIPTION

Gnomonia caryae var. **pecanae,** nov. var.

Peritheciis hypophyllis, pezizoideis, singulis vel 2–3 gregariis, plerumque inter pycnidia sparsis, sine rostris 300μ–350μ latis, 150μ–250μ altis; rostris 200μ–250μ longis, 50μ–60μ latis; pariebus perithecialibus apice et infra 2, lateribus 3–4 stratis cellularum compositis, atrobrunneis; ascis hyalinis, octosporis, poro apicali et pariebus tenuibus praeditis, cylindraceis, 45μ–50μ longis, 6μ–8μ latis; ascosporis rectis vel curvatis, hyalinis, guttulosis, uniseptatis, utrinque setosis, 22μ–28μ longis, 3μ–4μ latis.

Pycnidiis superficialibus, subglobosis, rufo-brunneis, 75μ–300μ latis; conidiis continuis, hyalinis, curvatis, fusiformibus, versus apicem attenuatis, 15μ–20μ longis, 2μ–3.5μ latis, conidiophoribus simplicibus, hyalinis, 1–2 septatis, 18μ–25μ longis, 3μ–5μ latis.

In foliis Hicoriae pecanae.

Perithecia hypophyllous, flask-shaped, single or in groups of twos or threes; usually found scattered among the older pycnidia; from 300μ to 350μ wide and 150μ to 250μ high without the beaks. Beaks from 200μ to 250μ long by 50μ to 60μ wide. Walls of perithecia 2 cells thick at bottom and top and 3 to 4 cells thick on sides; dark brown. Asci hyaline, 8-spored with pore at apical end, cylindrical, thin-walled, 45μ to 50μ by 6μ to 8μ. Ascospores 22μ to 28μ by 3μ to 4μ, straight or curved, hyaline, guttulate, and 1-septate with setae at both ends.

Pycnidia superficial, subglobose, reddish brown, 75μ to 300μ in diameter. Conidia 15μ to 20μ by 2μ to 3.5μ, continuous, hyaline, curved, fusoid, and attenuated toward the apex. Conidiophores simple, hyaline, 18μ to 25μ by 3μ to 5μ and 1- to 2-septate.

On leaves of *Hicoria pecan*.

Type specimens have been deposited in the Pathological Collections of the Bureau of Plant Industry, United States Department of Agriculture, at Washington; in the New York Botanical Garden; and in the Missouri Botanical Garden.

GROWTH ON ARTIFICIAL MEDIA

The liver-spot fungus was easily cultured by streaking corn-meal agar plates with either conidia or ascospores. Monospore cultures became macroscopic in about 10 days. From the beginning, the mycelium was very compact, light tan in color, and covered with short aerial hyphae. Production of conidia began in about 20 days, at the optimum temperature. The conidia were borne in pycnidia having the same characteristics as those on the host.

The fungus grows on a wide variety of artificial media. The best growth was made on corn-meal agar, when an area 10 cm in diameter was covered in 60 days. It fruited best on a combination of corn-meal and pecan or corn-meal and hickory agar, made by rolling pecan or hickory leaves and placing them in test tubes of corn-meal agar. In 10 days a few pycnidia appeared, and after 4 weeks the entire surface of the medium was covered with the fruiting bodies. On vegetable plugs, especially potato, an abundance of pinkish aerial hyphae was produced, but no conidia were present.

[12] WOLF, F. A. (See footnote 8.)

When kept in an incubator for 60 days on corn-meal and potato agar, the fungus grew best at a temperature of 24° C. At this temperature the maximum growth was 5½ cm. The mycelium was hyaline to light tan and mostly submerged, with little aerial hyphae. Little growth was made at 28° and none at 34° C. At 16° conidia and an abundance of white aerial hyphae were produced, although the growth was only 2 cm in diameter. No growth occurred at 12°.

During the course of hydrogen-ion studies, corn-meal agar medium with a pH range of from 4.0 to 8.5 was used. The optimum growth, an area 4 cm in diameter occurred between pH 5.8 and pH 7.0, whereas poor growth was made at pH 4.0 and pH 8.5. The general characteristics of the growth were the same at all of these hydrogen-ion concentrations. The mycelium was light brown, mostly submerged, with little or no aerial hyphae. Where growth was optimum the medium was discolored, changing from hyaline to light brown. Conidia were produced in all the cultures.

PATHOGENICITY

During the season of 1930 unsuccessful attempts were repeatedly made to produce liver spot by using ascospores from leaves as inocula. In 1931 all inoculations were made with conidia from leaves collected during the late fall of 1930 and from ascospore cultures. The imperfect and perfect stages of the fungus had previously been connected when perithecia of *Gnomonia* were found among the old pycnidia of the *Leptothyrium* stage and conidia were produced in cultures made from monospore cultures of the ascigerous stage. A mass of conidia was removed from culture tubes and from leaves by means of a scalpel and placed on both the dorsal and ventral surfaces of leaves of the Stuart, Van Deman, and Pabst varieties. The leaves were then enclosed in glassine bags for 48 hours. These inoculations were begun the 1st of May, while the leaves were young and before they had taken on a dark-green color. The inoculations were made in series every 2 weeks throughout the summer. The first infections were recorded September 9 on leaves that were inoculated July 28 with conidia taken from leaves and ascospore cultures. The results of inoculations on young and on older leaves were practically the same, although the percentage of infection was small. The later the inoculations were made, the higher was the percentage of infection; maximum infection occurred in October from inoculations made in September. The results with *Gnomonia* from the Stuart variety of pecan and the shagbark hickory (*Hicoria ovata*) were as follows: 58-percent infection was obtained on pecan with conidia from the hickory *Gnomonia* and 63 percent on hickory with conidia from the pecan *Gnomonia*, whereas 90-percent infection was obtained on pecan with conidia from the pecan *Gnomonia*, and 97 percent on hickory with conidia from the hickory *Gnomonia*. There was 48 percent *Gnomonia* infection on the pecan checks and 55 percent on the hickory checks. All infections were on the lower or dorsal surface of the leaves, indicating that the fungus enters the host through the stomata, since most of the stomata are on the lower surface. The period of incubation for infection was from 5 to 6 weeks for both old and young leaves,

indicating that the age of the leaves is an unimportant factor in the susceptibility to the liver-spot pathogene.

Temperature and humidity were doubtless the chief factors repressing infection earlier in the season, for natural infections of liver spot were present in the field during June and July.

VARIETAL SUSCEPTIBILITY

While most of the improved varieties of pecan are attacked by the liver-spot fungus, some are more susceptible than others. Among the most susceptible are the Stuart, Van Deman, and Pabst. In the vicinity of Shreveport, La., the Schley and Georgia Giant are highly resistant. Most of the native pecans in Texas are attacked, some being defoliated.

CONTROL

During the season of 1930, experiments were conducted on the Pabst variety in an effort to control the liver-spot disease. A block of trees was sprayed with one application of 4:6:50 bordeaux mixture on June 23. As the spring of 1930 was very early, most of the ascospores were discharged by May 1. The spraying was too late, for more than 50 percent of infection occurred. The experiment was repeated in 1931 on the Stuart variety, the application of 4:6:50 bordeaux mixture being made on May 27. The spring was later and the ascospores were not discharged until about May 10. There was not more than 2- to 3-percent infection from the liver-spot fungus on the sprayed trees, as compared with 75-percent infection on the unsprayed trees. The unsprayed trees were defoliated by September 1, whereas the sprayed trees held their foliage until December. These experiments indicate that in the vicinity of Shreveport, La., the fungus would be comparatively easy to control if the trees were sprayed during the latter part of May.

Sanitation may be an important factor in controlling the disease. If the leaves were destroyed by burning or if they were plowed under deeply enough in the fall or early spring, the early infection would undoubtedly be greatly reduced.

SUMMARY

Liver spot, a foliage disease of pecans, caused by *Gnomonia caryae* var. *pecanae*, nov. var., has been collected in Mississippi, Arkansas, Louisiana, and Texas.

The disease first appears on the lower surface of the leaves as circular brown dots resembling liver in color. Since the name "western sooty spot" now in use indicates that the leaf spot is black, which is not the case, the writer believes that "liver spot" would be a better term for this disease.

The fungus causes the greatest injury to trees of low vitality, but it also attacks vigorous trees and occasionally causes premature defoliation. It seems to thrive best in climates of high temperature and low rainfall.

The conidial stage of the fungus is considered as *Leptothyrium caryae* var. *pecanae*, distinct from *L. nervisedum* Cole. The perfect stage is considered as *Gnomonia caryae* var. *pecanae*, distinct from

G. setacea var. *macrospora* Ell. and Ev., from a 2-spored *Gnomonia* described by Matz in Florida, and from *G. caryae* Wolf.

The liver-spot fungus will grow on a variety of laboratory media, but fruits best on a combination medium of pecan or hickory leaves rolled and inserted in a test tube containing corn-meal agar. The optimum temperature for growth is 24° C. and the minimum is between 12° and 16°.

The writer was able to prove, according to Koch's rules, that the liver-spot disease is caused by *Gnomonia caryae* var. *pecanae*.

Varietal resistance was noted among pecans. The varieties Schley and Georgia Giant were highly resistant, whereas the Stuart, Van Deman, and Pabst varieties were very susceptible.

One application of 4:6:50 bordeaux mixture on May 27, 1931, gave almost perfect control of the liver-spot fungus.

FLOODING TO CONTROL ROOT-KNOT NEMATODES[1]

By LLOYD N. BROWN[2]

Agent, irrigation assistant, Division of Irrigation, Bureau of Agricultural Engineering, United States Department of Agriculture

INTRODUCTION

Use-of-water studies in the Sacramento-San Joaquin Delta were undertaken in 1924, and are still being continued. The delta, about 400,000 acres in area, is composed of peat and sedimentary soil, the latter type amounting to about 60 percent of the whole. The channels of the delta connect with an upper arm of San Francisco Bay and are subject to tidal action. This area, formerly a tidal swamp, was reclaimed by building levees around sections varying in size from a few hundred to several thousand acres. The ground surface of most of the enclosed areas is several feet below low-tide level. Irrigation water is withdrawn from the delta channels by siphoning it over the levees or carrying it under them in culverts, and is distributed to the land through a system of ditches. Excess water is collected in drainage canals and returned to the channels by pumping.

Work on the consumptive use of water, referred to above, consisted in part of growing crops on peat and sedimentary soil in typical moisture-requirement tanks. While this work was in progress, considerable difficulty was experienced with the root-knot nematode, *Heterodera marioni* (Cornu) Goodey. The damage caused by the nematodes in the delta is of considerable magnitude. Great areas of very valuable land are rendered unfit for the profitable production of potatoes, sugar beets, and other crops. No effective system of control is known. The nematode population can be reduced by replacing susceptible crops with a rotation of nonsusceptible crops such as barley or corn, or by burning off the surface layer of the peat, which is a very undesirable practice. Flooding had been suggested, but results obtained were neither conclusive nor in agreement. The method, however, appeared to offer possibilities and was definitely related to use-of-water studies.

[1] Received for publication Sept. 28, 1933; issued February 1934. The nematode control experiments reported herein, made in the period 1928–30, were incident to work on the consumptive use of water in the Sacramento-San Joaquin Delta, Calif. The project as a whole was carried on under a cooperative agreement subscribed to by the following parties: U.S. Department of Agriculture, Bureau of Public Roads, operating through the Division of Agricultural Engineering; State of California, Department of Public Works, operating through the Division of Engineering and Irrigation and the Division of Water Rights; University of California, Agricultural Experiment Station, operating through the Division of Irrigation Investigations and Practice. Reorganization has now (1933) resulted in the Federal agency becoming the Bureau of Agricultural Engineering, operating through its Division of Irrigation, and the State agency becoming the Division of Water Resources in the Department of Public Works.

[2] Gerald Thorne, Division of Nematology, Bureau of Plant Industry, U.S. Department of Agriculture, was detailed to advise and assist in planning the experiment. His services included two visits to King Island and microscopic examinations of soil and water samples. Jocelyn Tyler, Division of Entomology and Parasitology, University of California, did all greenhouse work and gave advice on numerous occasions. The California Delta Farms, Inc., through George Atherton, general manager, provided the site and water supply for the experiment and in other ways facilitated the installation. O. V. P. Stout, irrigation engineer, Bureau of Agricultural Engineering, who supervised the consumptive use of water studies, encouraged and forwarded the work from its inception to completion.

The larvae of *Heterodera marioni* are very slender, averaging 0.4 mm in length, and even on a dark background are barely visible to the unaided eye. Three conditions are necessary for their optimum

FIGURE 1.—*A*, Nematode-infested roots of pumpkin; *B*, nematode-infested roots of sugar beet.

growth—proper temperature, moisture, and air. Tyler [3] found that activity begins at about 55° F. and increases up to about 80°, after which it falls off rapidly. They multiply most rapidly when the sur-

[3] TYLER, J. DEVELOPMENT OF THE ROOT-KNOT NEMATODE AS AFFECTED BY TEMPERATURE. Hilgardia 7: 381–415, illus. 1933.

rounding soil is of proper moisture content for plant growth. The greatest numbers are found in the upper foot of soil, which is best aerated, although some may be found at a depth of several feet. In other words, they do best under conditions that are most favorable for the growth of plant roots. Some of the most common commercial plants subject to attack are potato, sugar beet, celery, tomato, melon, beans, cantaloupe, squash, cucumbers, peaches, and figs. Two views of roots infested with the nematodes are shown in figure 1.

REVIEW OF LITERATURE

Several writers have mentioned the possibility of controlling nematodes by flooding, but no precise work appears to have been done. Bessey,[4] after discussing the problem, sums up:

> Flooding, then cannot be recommended as a certain means of exterminating root-knot under all circumstances. Probably the soil should be flooded at least 25 days; in the laboratory the nematode larvae usually succumbed much sooner when isolated and placed in water.

Frandsen,[5] in reporting some flooding experiments, states:

> It has been suggested that fields might be rid of the worms by flooding them with water but our experiments indicate that at least 3 months submersion would be necessary to make this method effective.

Watson,[6] who has made some observations on flooding as a means of controlling root-knot, concludes:

> Nematodes can be greatly reduced in number, if not completely eradicated, by flooding. Indeed, in some fields usually flooded during part of the summer, as with tomato fields on the east coast, nematodes are seldom noticed. There is yet much to be learned as to the length of time a field should be covered with water, especially at different seasons, and as to the resistance of the eggs to water. Generally a week or two is considered necessary.

No flooding experiments were continued long enough to determine the time necessary to secure complete control.

It may be noted here that nematode larvae are not drowned by submergence in water, but supposedly succumb to exhaustion induced by their own activity, which is increased in water.

INSTALLATION OF PLOTS

A site, possessing the three prime requisites—unfailing water supply, adequate drainage, and heavy nematode infestation—was selected on King Island, Calif., in April 1928. King Island, a tract of about 3,300 acres, lies with its southeast corner about 7 miles west and 6 miles north of Stockton. Two microscopic examinations of the peat soil showed populations of *Heterodera marioni* estimated at 8 and 45 millions per acre. A crop of potatoes planted on the area 3 or 4 years previously had been a failure because of nematodes. Barley was grown on the area in 1926 and 1927. On May 8, 1928, barley about 2 feet high was cleared from the plot area. Many volunteer potatoes heavily invested with nematodes were found growing with the barley.

Twenty plots, each 12 feet square, arranged in a double row 10 plots long from east to west, or, in other words, 10 pairs of plots, were prepared. Each plot was enclosed by a watertight wall or curb made of tongue-and-groove redwood, extending 12 inches above and 18 inches below the ground surface. The tops of all the curbs were at the same

[4] BESSEY, E. A. ROOT-KNOT AND ITS CONTROL. U.S.Dept.Agr.Bur. Plant Indus. Bull. 217, 82 pp. illus. 1911.

[5] FRANDSEN, P. EELWORM PARASITES OF PLANTS. Calif. Comn. Hort. Mo. Bull. 5: 60-62. 1916.

[6] WATSON, J. R. CONTROL OF ROOT-KNOT, II. Fla. Agr. Expt. Sta. Bull. 159, pp. 29-44. 1921.

level throughout. Two views of the plots are shown in figure 2. The water supply was obtained from the channel nearby through 5-inch slip-joint pipe. The pipe discharged into a delivery box at the northeast corner of the plots. The north side of this box was built about 6 inches lower than the tops of the curbs enclosing the plots, thus providing a spillway which protected the plots from overflowing. The west end of the delivery box was connected to a wooden flume 10 inches deep and 12 inches wide built on the north side of the plots. Water was admitted to each pair of plots through holes in the north curb of the north plot of the pair, which was also the south side of the flume, and thence through auger holes in the middle curb to the south plot of the pair. With this set-up it was possible to hold water on all or any pair or pairs of the plots.

In addition to the natural infestation of nematodes, some badly affected roots of smartweed, *Polygonum acre* H.B.K., were buried a few inches deep at marked spots in each plot. This precaution was taken in order to have a small sample of soil for microscopic examination which was definitely known to contain nematodes.

After several trial floodings, flooding was continuous for the respective periods in each case, from July 18, 1928, until the end of the experiment, with the exception of a few unavoidable interruptions, none of which was for more than 24 hours' duration and most of them for considerably less.

FLOODING EXPERIMENTS ON KING ISLAND PLOTS

In preliminary experiments, peat soil infested with nematodes was submerged in small tanks. Several microscopic examinations of the soil up to and including one 3 months after flooding showed many living larvae. However, examination on the fourth month after flooding revealed only apparently exhausted larvae.

On November 29, 1928, slightly more than 4 months after flooding, samples of soil from the plots containing *Polygonum acre* roots were examined microscopically. No nematodes or eggs were found.

On January 22, 1929, after slightly more than 6 months of flooding, another similar set of samples was taken and examined microscopically. This examination also failed to disclose any nematodes or eggs.

On the same date two 6-inch and twelve 4-inch pots were filled with flooded soil and a tomato plant was placed in each. On March 1, 1929, after the plants had been in the greenhouse about 5 weeks, 9 galls were found on the roots in one 6-inch pot and 1 each in 4 of the 4-inch pots. The other plants appeared to be free from nematodes.

When the above-noted samples were taken, 6 plots were drained. On June 1, about 5 months after draining, these plots were planted with pumpkin seed. On July 5, 35 days later, the pumpkin roots showed many galls, microscopically determined to be caused by nematodes. Other plants growing in the plots, and susceptible to nematode attack, also showed galls.

From the foregoing observations it would appear that from 4 to 6 months' submergence will kill nematode larvae, but viable eggs remain and under favorable conditions these will hatch.

On July 18, 1929, 6 more plots were drained after having been under water for 12 months. On August 9 all plots were planted with pumpkin seed. Soil and pumpkin-root samples taken September 12 failed to show any nematodes when examined microscopically. However,

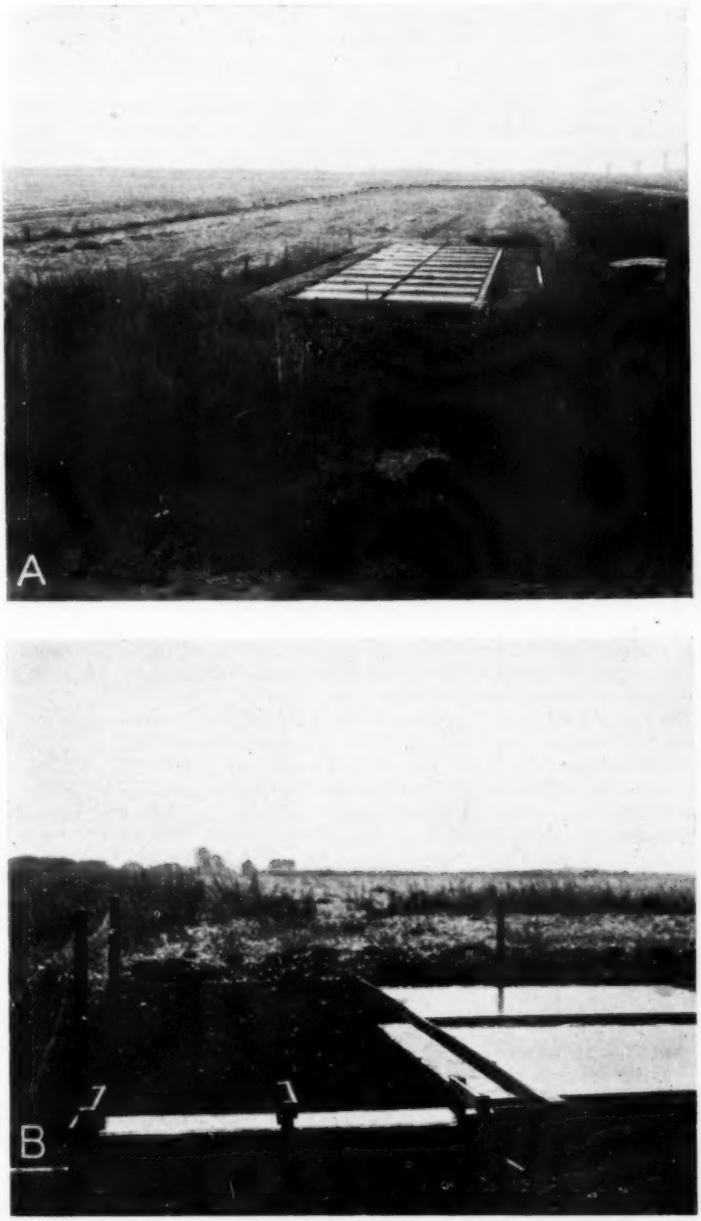

FIGURE 2.—*A*, General view of nematode plots on King Island, west from top of levee. Note drainage ditches. *B*, East end of nematode plots on King Island, showing supply pipe, receiving box, spillway, flume, and plots nos. 1 and 2.

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when pumpkin roots were dug out on October 9 some nematode galls were found. Hence, it would appear that after a year's flooding some of the nematodes had lived over in egg form, although apparently in greatly reduced numbers.

On June 4, 1930, 22½ months after flooding, 4 more plots were drained, and on June 18 these were planted to pumpkins. Roots of pumpkins were examined on July 10 and 26 and again in October, but in no case were any nematode galls found. *Polygonum acre* grew in the plots, but no evidence of nematodes was observed on the roots. Therefore it appears that all viable nematodes had hatched and the larvae died during 22½ months of flooding. Apparently between 12 and 22½ months of flooding was necessary to rid the land of nematodes. Under ordinary conditions this would probably be considered too long a period to be of practical value. However, under conditions similar to those prevailing in the Sacramento-San Joaquin Delta it may be practicable to flood for 2 years in order to rid the land of this pest, especially when prices of farm produce are low.

FLOODING EXPERIMENTS ON EMPIRE TRACT

The Empire tract, an island of about 3,000 acres of peat soil, lying just west of King Island, was flooded from about May 22 to September 22, 1929, in an effort to leach out alkali, kill weeds, and improve the soil. Previous to the flooding, four samples of soil badly infested with nematodes were obtained by pressing 9½-inch lengths of slipjoint pipe into the soil and then digging them out, thus removing a pipe full of soil. Two samples contained small sugar beets, the other contained *Polygonum acre* plants. The filled pipes were then buried with the upper ends even with the soil surface on the island to be flooded.

The pipes of soil were dug up after the 4 months of flooding. A microscopic examination of the material from 2 of the cylinders, 1 each of the sugar beet and *Polygonum acre* roots, failed to show any nematodes. The soil from the other 2 cylinders was placed in eighteen 4-inch flowerpots in a greenhouse February 11, 1930. On March 25 a *P. acre* plant in one pot had 5 galls, a *P. acre* plant in another pot had 2 galls, and a tomato plant had 4; the tomato plants in the other pots had respectively, 4, 4, 5, 6, 7, 9, 9, 9, 9, 10, 10, 13, 13, 17, 25, and 34 galls. These results are consistent with those obtained on the King Island plots, in indicating that 4 months' flooding will kill larvae but that viable eggs still remain.

SUMMARY AND CONCLUSIONS

Four months' submergence of soil containing the nematode *Heterodera marioni* killed the larvae, but the eggs remained viable. Flooding for 6 months gave approximately the same results as for 4 months. Nematodes appeared in soil flooded for a year, although apparently in greatly reduced numbers. After soil had been submerged for 22½ months no nematodes were found when plants were grown in it.

Therefore, it may be concluded that about 4 months' of submergence kills *Heterodera marioni* larvae, but the eggs are not killed until sometime between the end of the twelfth month and midway of the twenty-third month; hence, to rid nematode-infested soil by continuous flooding would entail the loss of 2 years' crops.

CYSTINE DEFICIENCY OF SOYBEAN PROTEIN AT VARIOUS LEVELS, IN A PURIFIED RATION AND AS A SUPPLEMENT TO CORN [1]

By CHARLES L. SHREWSBURY, *associate chemist*, and JOHN W. BRATZLER,[2] *senior student, Purdue University Agricultural Experiment Station and College of Agriculture*

INTRODUCTION

The extensive use of soybeans (*Soja max*) and soybean products in livestock rations, as well as in the dietary of man, makes information on the nutritive properties of this legume of importance. Mitchell and Smuts [3] recently published data which strongly indicate that a ration containing 10 percent of soybean protein (ether extracted soybeans) is deficient in cystine. The experimental period was short (5 weeks), and the rations were not readily consumed, resulting in very limited growth.

Csonka and Jones [4] have recently analyzed the glycinin from 10 varieties of soybeans for cystine and other amino acids. The glycinin from Manchu soybeans contained next to the highest amount of cystine (1.45 percent).

Soybeans are generally fed to livestock as a supplement to corn or other carbohydrate-rich feed. The experiments described in this paper were designed to reinvestigate the reported deficiency of soybean protein at a level of 10 percent, to determine whether a cystine deficiency existed at a protein level of 15 percent, and whether a ration made from corn and soybeans, such as is used in swine feeding, would exhibit a cystine deficiency.

EXPERIMENTAL PROCEDURE

Two methods of feeding were used in the experiments: In one series feed consumption was equalized; in the other the animals (rats) were allowed to eat ad libitum. In both series the animals were separated into pairs, one animal of a pair receiving the control ration and the other the cystine-supplemented ration. The animals of a pair were selected from stock shortly after weaning and were of the same age and sex, similar in weight, and from the same litter. Thus, the only difference in the two series was that in one case feed consumption was equalized, whereas in the other feed was available at all times. It was felt this would give some information as to the relative merits of the two methods of experimentation. The experimental period was 8 weeks.

The yellow corn and the Manchu variety of soybeans used in the experiments were obtained from the experimental swine farm and were of good quality. The soybeans were extracted with ether for several

[1] Received for publication Sept. 28, 1933; issued February 1934.

[2] A part of the material in this paper was submitted by the junior author to the School of Agriculture, Purdue University, as a thesis in partial fulfillment of the requirements for the bachelor of science degree.

[3] MITCHELL, H. H., and SMUTS, D. B. AMINO ACID DEFICIENCIES OF BEEF, CORN, OATS, AND SOYBEANS FOR GROWTH IN THE WHITE RAT. Jour. Biol. Chem. 95: 263–281. 1932.

[4] CSONKA, F. A., and JONES, D. B. DIFFERENCES IN THE AMINO ACID CONTENT OF THE CHIEF PROTEIN (GLYCININ) FROM SEEDS OF SEVERAL VARIETIES OF SOYBEAN. Jour. Agr. Research 46: 51–55. 1933.

days and dried. The yellow corn and ether-extracted soybeans had a crude protein content of 9.5 and 51.7 percent, respectively. The other materials used in the rations were of good quality. Table 1 gives the composition of the rations.

TABLE 1.—*Composition of rations*

Constituent	Percentage composition of ration no.—					
	261	262	264	265	259	260
Ether-extracted soybeans	11.5	11.5	29.0	29.0	20.0	20.0
Yellow corn	84.0	84.0				
Dextrin	2.5	2.25	54.0	53.75	63.0	62.75
Butter			8.0	8.0	8.0	8.0
Yeast			2.0	2.0	2.0	2.0
Cod-liver oil			2.0	2.0	2.0	2.0
Salts 6 [a]	2.0	2.0				
Osborne-Mendel salts [b]			4.0	4.0	4.0	4.0
NaCl			1.0	1.0	1.0	1.0
Cystine		.25		.25		.25
Crude protein from soybeans	5.94	5.94	14.99	14.99	10.34	10.34

[a] Limestone 10 parts, special steamed bone meal 10 parts, common salt 1 part.
[b] OSBORNE, T. B., and MENDEL, L. B. NUTRITIVE VALUE OF THE WHEAT KERNEL AND ITS MILLING PRODUCTS. Jour. Biol. Chem. 37: 572. 1919.

The cystine used in the experiments was obtained from the Eastman Kodak Co. and was labeled L-cystine. It was found to be 97.25 percent pure, based on its nitrogen analysis.

EXPERIMENTAL RESULTS

The supplementing effect of cystine on a purified ration containing 10 percent of soybean protein is seen by an examination of table 2.

TABLE 2.—*Supplementing effect of cystine on a purified ration (259) containing 10 percent of soybean protein when fed to rats, as indicated by their weights in grams*

[Experimental period 56 days]

FEED CONSUMPTION EQUALIZED

Item	Pair 1		Pair 2		Pair 3		Pair 4	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	43	40	42	40	46	44	40	47
Final weight	71	71	59	64	*86	102	*78	91
Total gain	28	31	17	24	40	58	29	44
Total feed	208	208	241	241	282	283	201	201

FEED CONSUMPTION UNRESTRICTED

Initial weight	38	36	34	34	30	29	35	33
Final weight	59	52	*47	58	*56	55	*46	74
Total gain	21	16	13	24	26	26	11	41
Total feed	216	218	114	118	135	123	122	180

* Died before the end of the experiment; pair mates receiving cystine were continued on and were all alive at the end of the experiment.

TABLE 2.—*Supplementing effect of cystine on a purified ration (259) containing 10 percent of soybean protein when fed to rats, as indicated by their weights in organs*—Continued

Item	Pair 5		Pair 6		Pair 7		Pair 8	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
FEED CONSUMPTION EQUALIZED								
Initial weight	42	40	39	38	47	47	45	43
Final weight	59	64	[a] 69	74	[a] 67	86	[a] 67	75
Total gain	17	24	30	36	28	39	22	32
Total feed	241	241	228	228	236	236	168	169
FEED CONSUMPTION UNRESTICTED								
Initial weight	30	29	36	35	35	33	46	46
Final weight	[a] 40	65	[a] 45	51	[a] 57	59	[a] 74	93
Total gain	10	36	9	16	22	26	28	47
Total feed	117	147	157	235	127	111	197	201

[a] Died before the end of the experiment; pair mates receiving cystine were continued on and were all alive at the end of the experiment.

The results of all the feeding trials have been summarized statistically according to the method of Student [5] in table 3. In the experiments in which feed consumption was equalized all of the pair mates receiving cystine in the ration made larger gains than the controls. Although the rations were consumed in considerable quantity, growth was relatively poor. Five of the control animals died before the end of 50 days, while none of the pair mates receiving cystine had succumbed by the end of the experiment. The weights and feed consumptions recorded in the table for the pair mates that died are for the last period preceding death. A marked difference was noted between the hair coats of the rats; the ones receiving cystine were smooth and well kept, while the controls had rough coats.

TABLE 3.—*Statistical results of experiments with cystine as a supplement in the diet of rats*

Item	Soybean protein 10 percent (ration no. 259), feed equalized	Soybean protein 10 percent (ration no. 259), feed unrestricted	Soybean protein 15 percent (ration no. 264), feed equalized	Soybean protein 15 percent (ration no. 264), feed unrestricted	Corn and soybean protein (ration no. 261), feed equalized	Corn and soybean protein (ration no. 261), feed unrestricted
Total mean difference in gain=M	[a]+9.6	[a]+11.1	[a]+12.1	[a]+10.5	[a]+0.17	[b]−9.9
Standard deviation S	4.95	12.5	6.31	14.1	6.03	21.4
Ratio $M/S=Z$	1.97	.92	1.93	.77	.028	.46
Probability P	.0006	.026	.0007	.041		.1332
Probable error	±1.17	±3.94	±1.48	±3.32	±.943	±5.0
Observation number	8	8	8	8	18	8

[a] +=Total mean difference in total gain in weight favors cystine-supplemented ration.
[b] −=Total mean difference in total gain in weight favors control ration.

The data obtained from the experiments in which the animals had unrestricted access to the feed cannot be said to establish definitely the cystine deficiency of soybean protein. Five pair mates receiving cystine made better gains than did the controls, two made about the same gain, and one did not gain so much. The mortality among the control animals was pronounced. Feed records in the case of rations

[5] ANONYMOUS. THE PROBABLE ERROR OF A MEAN. By Student. Biometrika 6: 1-25, illus. 1908.

of this type may, perhaps, be open to some question, since losses due to wastage are unavoidable.

The statistical treatment of the data reveals striking differences in the accuracy of the two feeding methods. The probability that the difference in gain in favor of the cystine-supplemented ration was due to chance was very small in the experiments in which feed consumption was equalized. The probable error of the result ± 1.17 is also quite small. (Probable error is in terms of grams of gain). However in the experiments in which access to feed was unrestricted the probability that the results were due to chance was larger ($P = 0.026$). The probable error was also larger, ± 3.94. It would seem that where unequal feed consumption might produce irregular gains a larger number of experiments would be required to establish a given result.

The relatively poor growth and high mortality obtained by feeding the purified ration containing 10 percent of soybean protein led to an investigation of the possible cystine deficiency of soybean protein fed at a level of 15 percent. This is nearer the amount of protein that is considered to be necessary for satisfactory growth of rats. The results of this work are presented in table 4.

TABLE 4.—*Supplementing effect of cystine on a purified ration (264) containing 15 percent of soybean protein when fed to rats, as indicated by their weights, in grams*

[Experimental period 56 days]

FEED CONSUMPTION EQUALIZED

Item	Pair 1		Pair 2		Pair 3		Pair 4	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	45	44	43	41	42	41	35	35
Final weight	97	104	90	98	91	110	* 66	77
Total gain	52	60	47	57	49	69	31	42
Total feed	453	453	401	401	390	390	169	169

FEED CONSUMPTION UNRESTRICTED

Initial weight	38	38	42	39	37	37	37	37
Final weight	103	109	96	135	93	99	* 74	65
Total gain	65	71	54	96	56	62	37	28
Total feed	405	355	407	442	327	347	220	179

FEED CONSUMPTION EQUALIZED

Item	Pair 5		Pair 6		Pair 7		Pair 8	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	39	36	41	39	42	41	41	40
Final weight	81	85	92	114	92	100	* 82	89
Total gain	42	49	51	75	50	59	41	49
Total feed	372	372	420	420	423	423	231	231

FEED CONSUMPTION UNRESTRICTED

Initial weight	37	37	48	46	39	38	37	35
Final weight	* 90	85	* 93	105	95	121	69	71
Total gain	53	48	45	59	56	83	32	36
Total feed	219	178	176	204	388	450	217	194

* Died before the end of the experiment; pair mates receiving cystine were continued on that diet and were all alive at the end of the experiment.

Although feed consumption was higher and growth was greater than when 10 percent of soybean protein was fed, a definite increase in rate of gain was obtained when cystine was included in the ration. The mortality was reduced when the level of soybean protein amounted to 15 percent of the ration. All the deaths that occurred were of animals on the unsupplemented control ration. The quality of the hair coat was better in the animals receiving the cystine supplement, as was the case when 10 percent of soybean protein was fed.

The supplementing effect of cystine on soybean protein could be deduced, perhaps, from the experiments in which the animals had unrestricted access to feed, since all but two of the pair mates receiving cystine made larger gains than the controls. In 6 of the 8 pairs of rats that had access to food at all times, the rat that consumed the most feed made the better gain.

A consideration of the statistical results demonstrates that the experiments in which feed consumption was equalized gave the most reliable results. The probable error of the mean result was higher and the probability that the result was due to chance was also higher in the series of experiments, in which unrestricted access to feed was allowed.

The possible significance of the cystine deficiency of soybeans in the feeding of livestock is interesting. It has been demonstrated [6] that a ration prepared from corn, soybeans, and minerals, in which soybeans furnish approximately 6 percent of protein, is not adequate for satisfactory growth of rats and swine. Cooking or roasting the soybeans in this ration results in a definite improvement in its growth properties. Since soybeans or soybean products are utilized in practice as a protein supplement to corn or other carbohydrate feed, it seemed worth while to determine whether cystine would supplement a ration likely to be used in farm practice. The results might help to explain the change that soybeans undergo during cooking or roasting. The results given in table 5 show that a cystine supplement does not improve the ration in which yellow corn is present.

[6] SHREWSBURY, C. L., VESTAL, C. M., and HAUGE, S. M. THE EFFECT OF YEAST AND CASEIN SUPPLEMENTS TO CORN AND SOYBEAN RATIONS WHEN FED TO RATS AND SWINE. Jour. Agr. Research 44:267-274, illus. 1932.

TABLE 5.—*Supplementing effect of cystine on a ration (261) of yellow corn and 5.94 percent of soybean protein when fed to rats, as indicated by their weights in grams*

[Experimental period 56 days]

FEED CONSUMPTION EQUALIZED

Item	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5		Pair 6	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	37	35	46	46	47	47	47	46	44	43	37	38
Final weight	95	89	129	137	113	119	122	130	114	115	105	97
Total gain	58	54	83	91	66	72	75	84	70	72	68	59
Total feed	384	384	553	553	466	466	469	469	467	467	405	405

Item	Pair 7		Pair 8		Pair 9		Pair 10		Pair 11		Pair 12	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	36	36	39	40	41	41	38	37	52	50	39	36
Final weight	107	99	117	115	155	163	119	122	146	139	95	97
Total gain	71	63	78	75	114	122	81	85	94	89	56	61
Total feed	432	432	466	466	626	626	470	468	534	533	384	385

Item	Pair 13		Pair 14		Pair 15		Pair 16		Pair 17		Pair 18	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	37	36	36	38	34	31	45	45	39	40	37	40
Final weight	125	124	117	114	116	114	122	116	124	131	123	120
Total gain	88	88	81	76	82	83	77	71	85	91	86	80
Total feed	501	503	478	475	442	447	506	501	501	502	489	487

FEED CONSUMPTION UNRESTRICTED

Item	Pair 1		Pair 2		Pair 3		Pair 4	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	52	52	36	35	35	35	47	47
Final weight	165	177	157	107	124	106	[a] 121	101
Total gain	113	125	121	72	89	71	74	54
Total feed	700	669	582	322	452	378	455	328

Item	Pair 5		Pair 6		Pair 7		Pair 8	
	Control	Cystine	Control	Cystine	Control	Cystine	Control	Cystine
Initial weight	49	51	52	53	35	35	34	35
Final weight	151	163	141	146	132	138	122	99
Total gain	102	112	89	93	97	103	88	64
Total feed	669	680	598	547	535	435	489	295

[a] Dead at end of 42 days; pair mate receiving cystine alive at end of 55 days.

In both series of experiments only 50 percent of the pair mates receiving cystine made larger gains than the controls. In the experiments in which feed consumption was equalized it was not possible to determine the degree of probability that the small difference in

growth in favor of the cystine-supplemented ration was due to chance, since Student's tables do not furnish probability figures for values of Z lower than 0.1. However, an inspection of the value of 0.1 ($P=0.3425$) shows that it is unlikely that the mean increase in gain in favor of the cystine supplemented ration is significant.

SUMMARY

The possible cystine deficiency of soybean protein fed at levels of 10 and 15 percent in a purified ration, and at a level of 6 percent as a supplement to corn, was studied by means of the paired-feeding method in which feed consumption was equalized and in similar experiments in which the animals (rats) had unrestricted access to feed.

Soybean protein (ether-extracted soybeans) was found to be definitely deficient in the amino acid cystine when the protein was fed at a level of 10 or 15 percent in a purified ration.

A ration made from corn, soybean protein, and minerals, such as is used in livestock rations, was not improved by the addition of L-cystine.

The advantages of the paired-feeding method in which feed consumption is equalized, in the determination of amino acid deficiency by means of nutrition experiments with rats, is demonstrated by these experiments, as compared with unrestricted consumption.

PLUMAGE AND EYE COLOR INHERITANCE IN THE SINGLE COMB RHODE ISLAND RED FOWL [1]

By D. C. WARREN, *professor of poultry husbandry*, and C. D. GORDON, *graduate assistant, Kansas Agricultural Experiment Station*

STANDARD COLOR OF BREED

An examination of a flock of Rhode Island Red fowls shows the color of the plumage and eyes to be highly variable. In the average flock there are birds varying from a light shade (approaching buff) to a deep mahogany or bay color. The eyes also show a wide variation in the amount of red pigment in the iris. These variations exist in spite of the fact that the breed has been selected for years for a deep red color of both plumage and iris. The wide range of intensity of pigmentation found in this breed would seem to suggest that the ideal standard color is rather complex in the nature of its inheritance.

The American Standard of Perfection [2] gives the description of the ideal bird of the Rhode Island Red breed as specified by the American Poultry Association. This standard requires that the Rhode Island Red be of a deep reddish bay color throughout the plumage of both sexes. It is desired that all sections of the plumage be as uniform in color as possible. The under fluff of the feathers should also be of the same color as the surface.

Tipping of black is permitted in the neck feathers of females. Black color is also desired in the primary and secondary flight feathers of both sexes. The upper web of the primary feathers should be red and the lower web largely black. In the secondaries the color is reversed, the lower web being red and the upper one largely black. The main tail feathers of both male and female are black. The sickle and covert feathers are also black or greenish black in the males. The occurrence of white in any section of the plumage and of black, except where specified, constitutes a defect in color.

Very little has been published on the inheritance of the color qualities of the Rhode Island Red. Bittenbender [3] recommended that both male and female be the same color, and he further mentioned the necessity of having a bar of black pigment in the back feathers of only one parent to assure rich, red plumage color in the offspring.

Dunn [4] suggested that the Rhode Island Red carries the recessive gene e^m which restricts melanic pigment to the neck, wings, and tail. The standard established for this breed has caused the melanic pigment to be reduced to a minimum in regions, except the flight and tail feathers.

[1] Received for publication July 24, 1933; issued February 1934. Contribution No. 76 from the Department of Poultry Husbandry, Kansas Agricultural Experiment Station.

[2] AMERICAN POULTRY ASSOCIATION. THE AMERICAN STANDARD OF PERFECTION . . . 1930 ed., 487 p., illus. n.p. 1930.

[3] BITTENBENDER, H. A. BREEDING RHODE ISLAND REDS FOR STANDARD TYPE AND EGG PRODUCTION. Iowa Agr. Expt. Sta. Bull. 202, pp. [11]–24, illus. 1922.

[4] DUNN, L. C. A GENE FOR THE EXTENSION OF BLACK PIGMENT IN DOMESTIC FOWLS. Amer. Nat. 56: 464–466. 1922.

Hays [5] in his proposed theory on the inheritance of color in the Rhode Island Red, suggested that three genes are responsible for the plumage pattern. The autosomal gene B for reddish-brown pigment, in combination with either a sex-linked gene L for color pattern and gold color or an autosomal gene E for the extension of melanic pigment, is responsible for the color phenotypes found in this breed. Hays did not attempt to account for the minor fluctuations which he suggests are probably due to the action of modifying factors.

The purpose of this study was to analyze, insofar as the available data will permit, the color inheritance in the Single Comb Rhode Island Red. The data upon which the analysis was made were taken from the Kansas Agricultural Experiment Station flock of this breed. The matings involved were made for the study of other characteristics in the fowl, but along with other records detailed color descriptions were also recorded for each individual. It was originally planned that this study would be preliminary to later ones where matings would be made for the specific purpose of the analysis of color inheritance. It now seems necessary to terminate the work on this project with the present paper.

MATERIALS AND METHODS

The stock used in this study had been handled and bred for some time as a production flock of Single Comb Rhode Island Reds, and an effort had been made to maintain fairly good standard qualities. Although there was considerable variation among individuals in this flock, the average color had been kept a good dark red.

In order to maintain uniformity and accuracy in classification of eye and plumage color, all descriptions were based on a set of color standards for each region considered.

The standards for feather color were the result of selection, after an examination of a large number of birds, of five gradations in color as being representative of the range of feather colors found in this breed. These feather standards served for classifying both surface and under color. The standard was made by mounting on a white cardboard samples of plucked feathers. Grade 1 was the lightest shade of color while the successive grades indicated increasing intensity of color. The standard colors of plumage and the standard for eye color were those used in a previous experiment by the senior writer.[6]

The descriptions of primary and secondary wing feathers follow a grading system based on the amount of black pigment found on the surface of the feathers of this region. The second full-length primary and the second secondary flight feathers were used for recording the amount of black. The numbers 1 to 6 are used to designate increasing amounts of black. Grade 6 occurred so infrequently that it was included in grade 5 in the summaries, thus only five grades were required in the tables. The range of variations found is shown in figure 1.

The eye color grades were based on the amount of red pigment in the iris. The iris color of each bird was compared with a color chart

[5] HAYS, F. A. INHERITANCE OF PLUMAGE COLOR IN THE RHODE ISLAND RED BREED OF DOMESTIC FOWL. Genetics 11: [355]–371. 1926.

[6] WARREN, D.C. THE INHERITANCE OF RHODE ISLAND RED CHICK DOWN-COLOR VARIATIONS AND THEIR RELATION TO COLOR VARIATIONS IN ADULT PLUMAGE. Jour. Agr. Research 39: 781–794, illus. 1929.

painted from representative specimens of grades of pigmentation. The grades ranged from an iris with practically no red to one showing a deep reddish bay color.

All descriptive observations were made at maturity (6 months of age), and the birds were graded according to the color standards for the respective regions. In order to secure uniformity in grading, all descriptions were made under natural lighting conditions and by the same person. In the description of the plumage color, the back, hackle, and breast were graded separately. In the study of under color, only the shade of red was considered.

Rather than preserve whole skins from so large a number of birds, feather samples from different regions of the plumage were preserved on cards as previously described by Warren.[7] Complete records were kept on all birds available for the breeding seasons 1927 to 1930, inclusive, totaling over 3,000 individuals.

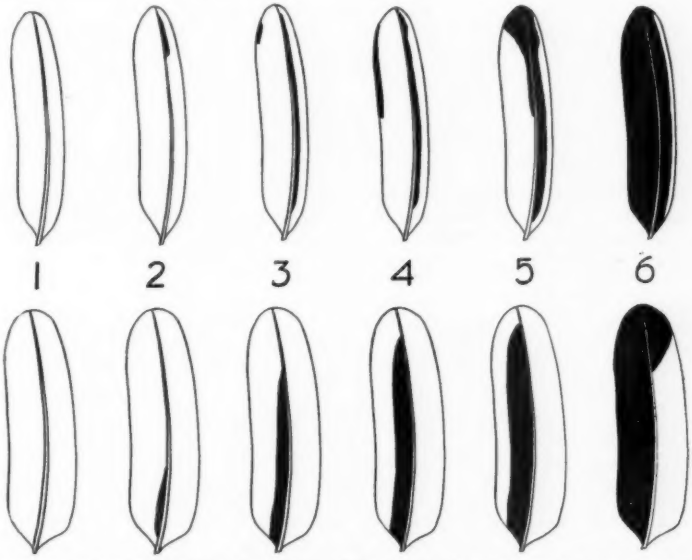

FIGURE 1.—Grade-number standards for variations in the amount of black pigment in the primary (upper series) and secondary (lower series) wing feathers of adult Rhode Island Red chickens.

RESULTS

In the study of color inheritance in various regions of the plumage the results were summarized on the basis of parental color combinations. The color grades of the offspring from the various paternal and maternal grades are shown in table 1. This made it possible to compare the results of mating the lightest colored female with the lightest colored male and darkest color with darkest color as well as reciprocal matings between color extremes. Because of the fact that birds in the extreme grades occurred infrequently and were mated

[7] WARREN, D. C. See footnote 6.

still less frequently, it was not possible to present the results of all color combinations. The various combinations of color grades were presented in the following tables insofar as they were available:

TABLE 1.—*Mean results from all available combinations of parental plumage and eye color grades*

BACK PLUMAGE

Sires' color grade (plumage)	Mean grade for daughters when the dams' color grade was—					Mean	Mean grade for sons when the dams' color grade was—					Mean
	1	2	3	4	5		1	2	3	4	5	
5	.....	2.98	3.19	3.35	.....	3.22	.....	4.44	4.33	4.43	.....	4.38
4	a2.75	2.71	2.99	3.10	3.61	3.07	a3.87	3.88	4.17	4.05	4.11	4.07
3	.....	.....	2.89	2.97	.....	2.94	.....	.....	a4.05	4.15	.....	4.12

BREAST PLUMAGE

5	.....	a2.44	a2.44	2.75	.....	2.66	.....	3.38	a3.91	3.58	.....	3.65
4	2.08	2.27	2.52	2.95	3.41	2.61	2.77	3.23	3.42	3.55	3.46	3.37
3	.....	2.22	2.43	2.41	.....	2.33	.....	2.97	3.17	3.34	.....	3.15
2	a1.18	2.17	2.07	2.45	.....	2.14	a2.50	2.55	2.90	2.91	.....	2.79

HACKLE PLUMAGE

5	.....	a3.22	3.60	3.68	.....	3.59	.....	a3.88	3.96	a4.17	.....	4.00
4	.....	a2.89	3.31	3.32	a3.93	3.32	.....	a3.29	3.77	3.82	a4.22	3.79
3	.....	3.19	3.18	3.37	3.46	3.27	.....	3.38	3.60	3.66	3.47	3.59
2	.....	2.88	3.13	3.26	.....	3.14	.....	3.35	3.26	3.56	.....	3.41

UNDER PLUMAGE

5	.....	a2.42	2.33	a2.64	.....	2.45	.....	a3.09	a2.89	a2.60	.....	2.91
4	a2.39	2.48	2.46	2.81	3.09	2.69	2.46	2.71	2.76	2.77	2.98	2.76
3	.....	2.49	2.69	2.72	.....	2.61	.....	2.85	3.10	2.96	.....	2.96
2	a2.59	2.30	2.54	2.61	.....	2.50	a2.75	2.72	2.64	2.81	.....	2.74

BLACK IN PRIMARIES

5	a2.73	2.68	2.93	3.21	.....	2.84	a3.33	3.42	4.06	4.26	.....	3.68
4	2.39	2.49	2.83	a2.90	.....	2.63	a3.33	3.42	4.02	a3.72	.....	3.61
3	a2.05	2.54	2.66	a2.88	.....	2.60	a3.00	3.20	3.39	a4.00	.....	3.35
2	a1.82	1.93	2.14	a2.83	.....	2.04	a2.40	2.54	2.92	3.78	.....	2.75

BLACK IN SECONDARIES

5	3.14	3.11	3.33	3.52	a3.90	3.35	a3.95	4.06	4.09	4.32	a4.19	4.13
4	2.30	2.82	2.92	3.18	3.31	2.87	3.23	3.50	3.87	4.33	a4.15	3.81
3	a2.39	2.18	2.16	a2.46	.....	2.27	2.62	3.49	3.35	a3.50	.....	3.22
2	1.91	2.22	2.52	a2.91	a2.63	2.39	a2.53	a2.86	3.38	a3.67	a3.91	3.20
1	1.66	a1.57	a2.31	.....	.....	1.87	a1.92	a2.21	a2.73	.....	.....	2.22

EYE COLOR

6	.....	.....	a9.39	9.23	.....	9.27	.....	.....	a8.83	8.74	.....	8.75
5	a9.40	9.29	9.88	9.56	9.63	9.55	a10.00	9.52	9.85	9.82	10.00	9.80
4	a8.69	9.22	9.38	9.52	9.36	9.39	a9.33	9.55	9.44	9.64	9.69	9.47
3	a8.75	9.27	9.09	9.46	a9.30	9.24	a8.54	9.41	9.07	9.24	a9.27	9.17

a These means are for classes of less than 30 individuals.

The inheritance of the shade of red color was studied in four regions of the plumage: Back (mid-dorsal region); breast; hackle, which is the collar of long pointed feathers covering the base of the neck; and the under surface, which includes the fluff at the base of the feather. The under color was that of the mid-dorsal region. There is frequently considerable contrast in the shade of red in these different regions, and for that reason they were considered separately. The breast is frequently lighter in color while the hackle color may be either lighter or darker than the general surface color. The under color is usually considerably lighter than that of the surface.

The inheritance of the amount of black in the wing feathers was studied, and the results from the primary and secondary wing feathers were tabulated separately. In addition to the studies on plumage color the inheritance of eye color was also considered.

INHERITANCE OF BACK COLOR

Since the back color of the males averaged somewhat darker than that of the females, it was necessary to tabulate separately the results from the two sexes. Any study of back color is complicated by this sexual dimorphic condition, because a male and female of the same genetic make-up are not the same color.

The results from the matings of individuals varying in back color are shown in tables 1 and 2. In table 1 are given the mean grades of sons and daughters from varying parental grades for all surface regions. These condensed results show the general trends but are not satisfactory for genetic analyses. In table 2 is presented the distribution in the various matings of the offspring among the five grades of back color.

TABLE 2.—*Distribution of offspring and mean results from all available parental combinations of grades of plumage and eye color*

BACK PLUMAGE

Parental color grade	Distribution of daughters in color grades indicated						Distribution of sons in color grades indicated					
	1	2	3	4	5	Mean grade	1	2	3	4	5	Mean grade
♂ ♀												
5×4	.....	16	71	72	.....	3.35	.....	.....	4	57	53	4.43
5×3	.....	21	142	63	.....	3.19	.....	.....	6	99	60	4.33
5×2	1	9	35	10	.....	2.98	.....	.....	3	13	18	4.44
Total or average	1	46	248	145	.....	3.22	.....	.....	13	169	131	4.38
4×5	.....	2	60	94	2	3.61	.....	1	7	101	24	4.11
4×4	2	65	292	113	1	3.10	.....	1	48	272	70	4.05
4×3	6	77	228	85	.....	2.99	.....	2	28	203	87	4.17
4×2	6	62	92	19	1	2.71	1	.....	35	100	20	3.88
4×1	.....	6	13	1	.....	2.75	.....	.....	2	13	.....	3.87
Total or average	14	212	685	312	4	3.07	1	4	120	689	201	4.07
3×4	.....	15	40	13	.....	2.97	.....	.....	4	39	12	4.15
3×3	.....	8	23	4	.....	2.89	.....	.....	2	14	3	4.05
Total or average	.....	23	63	17	.....	2.94	.....	.....	6	53	15	4.12

TABLE 2.—*Distribution of offspring and mean results from all available parental combinations of grades of plumage and eye color*—Continued

BREAST PLUMAGE

Parental color grade ♂ ♀	Distribution of daughters in color grades indicated						Distribution of sons in color grades indicated					
	1	2	3	4	5	Mean grade	1	2	3	4	5	Mean grade
5×4	7	30	25	21	1	2.75	.....	5	21	31	5	3.58
5×3	5	9	9	4	.....	2.44	.....	1	6	10	6	3.91
5×2	.....	6	2	1	.....	2.44	.....	1	4	2	1	3.38
Total or average	12	45	36	26	1	2.66	.....	7	31	43	12	3.65
4×5	.....	6	25	32	.....	3.41	1	2	15	17	2	3.46
4×4	17	62	102	78	2	2.95	.....	24	70	99	21	3.55
4×3	27	107	96	32	1	2.52	1	25	94	78	20	3.42
4×2	50	130	89	19	.....	2.27	.....	45	117	86	9	3.23
4×1	8	18	11	.....	.....	2.08	.....	11	15	4	.....	2.77
Total or average	102	323	323	161	3	2.61	2	107	311	284	52	3.37
3×4	14	84	60	12	.....	2.41	.....	21	59	52	8	3.34
3×3	5	83	54	8	.....	2.43	1	19	63	25	8	3.17
3×2	34	125	74	6	.....	2.22	1	48	79	31	7	2.97
Total or average	53	292	188	26	.....	2.33	2	88	201	108	23	3.15
2×4	5	32	22	6	.....	2.45	1	16	27	9	2	2.91
2×3	11	45	16	.....	.....	2.07	.....	13	18	9	.....	2.90
2×2	8	20	13	1	.....	2.17	.....	21	13	4	.....	2.55
2×1	14	3	.....	.....	.....	1.18	.....	5	2	1	.....	2.50
Total or average	38	100	51	7	.....	2.14	1	55	60	23	2	2.79

HACKLE PLUMAGE

5×4	.....	.....	11	23	.....	3.68	.....	1	3	11	9	4.17
5×3	.....	1	33	50	1	3.60	.....	1	13	46	12	3.96
5×2	.....	.....	7	2	.....	3.22	.....	.....	2	5	1	3.88
Total or average	.....	1	51	75	1	3.59	.....	2	18	62	22	4.00
4×5	.....	.....	5	19	3	3.93	.....	.....	.....	7	2	4.22
4×4	4	28	139	120	5	3.32	.....	11	47	123	31	3.82
4×3	.....	14	133	86	.....	3.31	.....	3	55	84	23	3.77
4×2	.....	6	19	3	.....	2.89	.....	1	8	5	.....	3.29
Total or average	4	48	296	228	8	3.32	.....	15	110	219	56	3.79
3×5	.....	.....	21	18	.....	3.46	.....	1	15	16	.....	3.47
3×4	.....	12	154	118	.....	3.37	.....	15	84	120	29	3.66
3×3	.....	27	203	83	.....	3.18	.....	13	117	140	26	3.60
3×2	.....	13	57	30	1	3.19	.....	8	29	33	1	3.38
Total or average	.....	52	435	249	1	3.27	.....	37	245	309	56	3.59
2×4	.....	4	98	41	.....	3.26	.....	1	53	64	2	3.56
2×3	.....	13	69	27	.....	3.13	.....	18	43	29	7	3.26
2×2	2	11	35	8	.....	2.88	.....	9	22	15	6	3.35
Total or average	2	28	202	76	.....	3.14	.....	28	118	108	15	3.41

TABLE 2.—*Distribution of offspring and mean results from all available parental combinations of grades of plumage and eye color*—Continued

UNDER PLUMAGE

Parental color grade ♂ ♀	Distribution of daughters in color grades indicated						Distribution of sons in color grades indicated					
	1	2	3	4	5	Mean grade	1	2	3	4	5	Mean grade
5×4		6	7	1		2.64		2	3			2.60
5×3		14	7			2.33		4	12	2		2.89
5×2		7	5			2.42			10	1		3.09
Total or average		27	19	1		2.45		6	25	3		2.91
4×5		15	74	25		3.09		12	71	10		2.98
4×4	4	50	107	23		2.81	4	39	88	14		2.77
4×3	7	69	65	4		2.46	1	31	48	11		2.76
4×2	1	50	46	1		2.48		34	64	4		2.71
4×1	1	15	12			2.39	4	22	24	2		2.46
Total or average	13	199	304	53		2.69	9	138	295	41		2.76
3×4	2	40	73	9		2.72		12	97	7		2.96
3×3	11	83	185	15		2.69		22	137	43		3.10
3×2	11	155	160	8		2.49	1	61	170	24		2.85
Total or average	24	278	418	32		2.61	1	95	404	74		2.96
2×4		70	82	8		2.61		26	97	2		2.81
2×3		35	35	2		2.54		29	37	4		2.64
2×2	7	73	45			2.30		30	73	1		2.72
2×1		7	10			2.59		2	6			2.75
Total or average	7	185	172	10		2.50		87	213	7		2.74

BLACK IN PRIMARIES

5×4		13	39	17	6	3.21		2	11	6	27	4.26
5×3	1	43	82	15	10	2.93		3	24	22	35	4.06
5×2	8	104	105	27	6	2.68	2	47	58	69	36	3.42
5×1		6	7	2		2.73		1	5	2	1	3.33
Total or average	9	166	233	61	22	2.84	2	53	98	99	99	3.68
4×4	2	15	22	8	3	2.90		8	13	10	16	3.72
4×3	3	25	32	5	7	2.83		4	11	25	20	4.02
4×2	8	58	41	6	4	2.49	1	22	43	41	18	3.42
4×1	5	22	10	1	3	2.39		9	7	4	7	3.33
Total or average	18	120	105	20	17	2.63	1	43	74	80	61	3.61
3×4	3	20	28	14	2	2.88	2	3	6	18	18	4.00
3×3	4	44	38	12	3	2.66	1	23	21	25	17	3.39
3×2	13	101	97	15	3	2.54	5	57	52	54	26	3.20
3×1	5	9	4	1		2.05	2	7	3	3	4	3.00
Total or average	25	174	167	42	8	2.60	10	90	82	100	65	3.35
2×4		7	20	2		2.83		7	5	8	12	3.78
2×3	15	57	21	4		2.14	3	29	31	16	6	2.92
2×2	58	206	35		1	1.93	19	105	49	26	10	2.54
2×1	3	7	1			1.82		7	2	1		2.40
Total or average	76	277	77	6	1	2.04	22	148	87	51	28	2.75

TABLE 2.—*Distribution of offspring and mean results from all available parental combinations of grades of plumage and eye color*—Continued

BLACK IN SECONDARIES

Parental color grade ♂ ♀	Distribution of daughters in color grades indicated						Distribution of sons in color grades indicated					
	1	2	3	4	5	Mean grade	1	2	3	4	5	Mean grade
5×5	.....	1	7	16	6	3.90	1	1	4	7	14	4.19
5×4	2	4	48	35	13	3.52	1	2	8	26	40	4.32
5×3	4	36	162	89	19	3.33	1	8	27	55	60	4.09
5×2	13	10	39	23	12	3.11	3	2	14	31	32	4.06
5×1	.....	7	20	8	2	3.14	2	.....	3	9	8	3.95
Total or average	19	58	216	171	52	3.35	8	13	56	128	154	4.13
4×5	4	1	16	3	8	3.31	2	.....	3	3	12	4.15
4×4	4	14	75	36	5	3.18	.....	3	12	45	56	4.33
4×3	29	64	143	69	14	2.92	10	23	58	89	99	3.87
4×2	16	29	51	28	5	2.82	11	14	27	34	30	3.50
4×1	32	37	36	10	4	2.30	13	14	17	28	16	3.23
Total or average	85	145	321	146	36	2.87	36	54	117	199	213	3.81
3×4	6	4	12	1	1	2.46	2	.....	1	8	1	3.50
3×3	15	18	13	5	.....	2.16	2	4	23	18	4	3.35
3×2	13	9	12	4	.....	2.18	6	3	6	17	9	3.49
3×1	12	8	19	5	.....	2.39	8	5	15	4	2	2.62
Total or average	46	39	56	15	1	2.27	18	12	45	47	16	3.22
2×5	1	3	3	.....	1	2.63	1	.....	3	2	5	3.91
2×4	2	6	8	4	2	2.91	2	1	8	5	8	3.67
2×3	18	20	32	7	5	2.52	7	8	17	13	16	3.38
2×2	12	16	19	2	.....	2.22	6	6	7	6	4	2.86
2×1	14	12	5	3	.....	1.91	9	9	3	5	4	2.53
Total or average	47	57	67	16	8	2.39	25	24	38	31	37	3.20
1×3	5	10	9	2	.....	2.31	2	5	4	3	1	2.73
1×2	8	4	2	.....	.....	1.57	6	2	3	3	.....	2.21
1×1	19	10	5	1	.....	1.65	13	4	4	2	1	1.92
Total or average	32	24	16	3	.....	1.87	21	13	11	8	2	2.22

EYE COLOR

6×5	1	15	22	19	7	9.23	3	13	16	3	3	8.74
6×4	.....	3	6	8	1	9.39	.....	3	1	2	.....	8.83
Total or average	1	19	28	27	8	9.27	3	16	17	5	3	8.75
5×6	1	4	30	24	14	9.63	.....	3	12	18	18	10.00
5×5	5	20	64	75	22	9.56	.....	12	48	56	43	9.82
5×4	1	9	51	96	47	9.88	1	13	44	78	45	9.85
5×3	1	18	25	37	4	9.29	.....	10	24	33	8	9.52
5×2	.....	3	13	13	1	9.43	1	1	.....	3	5	10.00
Total or average	8	54	183	245	98	9.55	2	39	128	188	119	9.80
4×6	1	15	41	22	14	9.36	1	7	20	28	15	9.69
4×5	3	29	69	76	34	9.52	1	14	53	69	26	9.64
4×4	4	37	74	82	24	9.38	4	28	54	72	22	9.44
4×3	1	15	34	20	7	9.22	1	8	29	26	13	9.55
4×2	2	5	6	2	1	8.69	.....	2	6	2	2	9.33
Total or average	11	101	224	202	80	9.39	7	59	162	197	78	9.47
3×6	.....	6	5	6	3	9.30	.....	4	5	4	2	9.27
3×5	2	14	31	39	12	9.46	1	20	25	30	6	9.24
3×4	.....	37	54	30	10	9.09	4	26	30	18	11	9.07
3×3	.....	11	19	14	5	9.27	2	5	12	7	8	9.41
3×2	1	4	4	3	.....	8.75	.....	6	4	1	.....	8.55
Total or average	3	72	113	92	30	9.24	7	61	76	60	27	9.17

The usual method of ascertaining in the fowl whether sex-linked factors are involved in a genetic make-up is to compare the female offspring from reciprocal matings. The sexual dimorphism encountered here makes this somewhat difficult. The means would indicate that males and females of the same genotype differ about one grade, the males being the higher. Selecting an average male (grade 4) and an average female (grade 3) and comparing the means when they were mated to the full range of grades of the opposite sex would supply some evidence as to whether sex-linked factors are involved. Since the female bird has only one sex chromosome and thus must receive it from her father she will show the influence of the sex-linked factors which he carries. Thus where the males are of a variable constitution, the daughters should show a greater range of variability than those from the cross where the females are of varying constitution. The mean grades of daughters from the mating of grade 3 females to the range of grades of males are no more variable than are those of daughters from the matings of grade 4 males to females of the different grades. Further evidence may be obtained from the comparison of the reciprocal mating of the most extreme—the darkest males with the lightest females and the lightest males with the darkest females. These combinations were 5♂×2 ♀ and 3♂×4 ♀, and the former had daughters averaging 2.98, and the latter 2.97 in color grade. Thus there appears to be no evidence that sex-linked factors are involved in producing the color variations on the back of the Rhode Island Red.

The rather consistent gradation of the means (for back color) shown in table 1 indicates somewhat strongly that variations in the surface color are inherited. The males used in the matings were of the grades 3, 4, and 5, while the females were distributed in all five classes. As the grade of the male decreased when mated to one grade of females, the mean of the offspring decreased. The same results were obtained from the different grades of females.

Although the evidence for heritability of the shade of red on the back appears to be good, it seems that the mode is not simple. The wide range of color grades among the off-spring in all crosses recorded in table 2 signifies that a number of determiners are probably responsible for the standard color.

INHERITANCE OF BREAST COLOR

The plumage of the breast region is usually lighter in color than that of the rest of the body (table 1). A comparison of the means of offspring from all available matings for back color and breast color shows that the males manifest a slightly greater difference in the two regions than the females.

Since the males were somewhat darker than the females with respect to breast color, the sexes were recorded separately. In table 2 the reciprocal crosses, grade 4♂× grade 2 ♀ and grade 2♂× grade 4♀, show the female offspring from the lighter males to be slightly darker than those from the darker males. Since this is the reverse of what would be expected from sex-linked factors, it may be stated that the sex chromosomes probably carry no genes affecting color in this region. There is also a slight difference in the mean of the males from these two combinations of matings, and this is not to be expected in cases of sex linkage. The difference in the means of offspring color from

these reciprocal matings is probably due to individual differences in the parent stock.

From the data in table 2 it is evident that the range of offspring from all available matings is relatively wide. In matings of grade 5 ♂ with grade 4 ♀ all five grades of female and four grades of male offspring were produced. This indicates that several factors are involved, and that many of the parents were not homozygous for the dark breast which they carried. Since the means of offspring from the different matings (table 2) seem to be lower than, rather than intermediate to, the parent grades, the major factors for light pigmentation are probably partially dominant to those for dark pigmentation.

TABLE 3.—*Coefficient of correlation of color intensity in various sections of plumage*

Sections of plumage	Number of birds	Region A		Region B		Correlation coefficient
		Mean	Standard deviation	Mean	Standard deviation	
Back (A) and breast (B)	1,022	3.11	0.691	2.41	0.835	0.787±0.008
Back (A) and hackle (B)	1,022	3.11	.691	2.24	.610	.660± .012
Back (A) and secondaries (B)	1,022	3.11	.691	2.84	1.041	−.013± .021
Primaries (A) and secondaries (B)	1,022	2.53	.903	2.84	1.041	.679+ .012

The data in table 3 show a positive correlation of 0.787 ± 0.008 between the grades of breast and back color. The probable explanation of this correlation is that the same major genetic factors control color in these two regions, although minor factors might cause some independent variation. The fact that the breast is usually lighter than other regions may be due to a general pattern factor.

INHERITANCE OF HACKLE COLOR

The difference found between the intensity of color in the hackle of the two sexes was less than that observed in the sections of the plumage previously discussed. From the values in table 2 it may be noted that when fowls in grades 4 and 5 were mated together they produced very few offspring lighter than grade 3, whereas when both parents were in grade 2 they produced several dark offspring. These results indicate that light color is dominant over dark since the heterozygotes would give the dominant color a wider range. The more restricted distribution may be the result of fewer factors being involved than in the case of the other regions studied. The inheritance of hackle color is probably dependent on more than one pair of factors, since no simple ratios can be assigned to the results.

An examination of the means from reciprocal matings, grade 5 ♂ × grade 3 ♀ and 3 ♂ × 5 ♀, or 4 ♂ × 2 ♀ and 2 ♂ × 4 ♀, show no indication of sex linkage. In one case the darker male gives the darker female offspring, and in the other case the darker male produces the lighter female offspring. These results indicate that genes in the sex chromosomes are not responsible for the variations in color of the hackle.

A positive correlation of 0.660 ± 0.012 (table 3) was found between the hackle and back color. The relatively high association of color intensity in these two regions would indicate that any existing differences are due to pattern factors. Another explanation of the correlation is that the same factors are responsible for the color of both regions and there are few modifying factors present to cause variations.

INHERITANCE OF UNDER COLOR

The study of inheritance of under color is based primarily on the red pigmentation in the fluff of the feathers on the back and does not take into consideration the black or white in this region. The variety of colors found in this part of the feather makes it very difficult to grade this region and may account for some of the irregular results obtained. The males seem to be slightly darker than the females (table 2), but there appears to be little order to the mean grade of offspring from different grades of males.

There is so little difference in average color grade from the different parental combinations that the evidence for inheritance of variations in under color is not good. These results may be due to unidentified environmental factors and also may be due to the narrow range of variation found. The narrow range in average color grade from the different parental combinations is evident from the results in table 2, there being little difference in the mean of offspring from grade 5 males and that of grade 2 males. There was a general tendency for the under color to vary with the surface color.

INHERITANCE OF BLACK IN PRIMARY AND SECONDARY WING FEATHERS

The major genetic factor considered in the inheritance of black in the primary and secondary wing feathers is the e^m factor of Dunn.[8] This factor acts by restricting the expression of melanic pigment to the extremities and is commonly known as the Columbian pattern. Dunn believes that its normal allelomorph is extended black. The variations studied here are probably due to modifying factors influencing the degree of restriction of black in the flight feathers. The amount of black in the web of the feathers of these two regions varies widely; some feathers are practically without black, while others are almost entirely black as seen in figure 1 which shows the range of the variations. The grade 6 occurred so infrequently that the few were included in grade 5 in the tables.

Upon examination of the means of offspring recorded in table 2 it is seen that there is more black in the secondaries than in the primaries. This difference may be due to a pattern factor or to a different combination of the factors affecting each region. Sexual dimorphism necessitated the recording of the sexes separately.

Most of the matings summarized in table 2 show a wide range of grades of offspring. This fact would indicate the possibility that several factors are responsible for the inheritance of black pigment in these two regions. The means of the offspring from the various matings, as recorded in table 2, are generally intermediate between the two parental grades, thus indicating a blending type of inheritance without much indication of dominance of either the lack or excess of melanic pigment. The possibility of the same factors controlling color in primaries and secondaries is supported by the correlation coefficient of 0.679 ± 0.012 between the degree of black in the two sections of the wing. The correlation-coefficient value is given in table 3.

The data in table 2 show the amount of black in the secondaries of the female offspring from the combination, grade 2 ♂ × grade 5 ♀ to be less than that from the combination, grade 5 ♂ × grade 2 ♀. This might be taken as evidence for sex linkage in the secondaries,

[8] DUNN, L. C. See footnote 4.

but it is rather doubtful that this could be the case after an examination of crosses 1 ♂×3 ♀, 3 ♂×1 ♀, 2 ♂×4 ♀, and 4 ♂×2 ♀ in which the female offspring do not seem to follow the mean of the male parent. The means of the male offspring in these reciprocal crosses differ to such an extent as to make more questionable the possibility of sex linkage. The means of female offspring from reciprocal matings 4 ♂×2 ♀, 2 ♂×4 ♀, and 1 ♂×3 ♀, 3 ♂×1 ♀ (table 2) show no indications of sex linkage in the primary wing feathers.

The means of the offspring from all mating combinations recorded in table 2 indicate that excess black in these two areas is inherited, because the means seem to recede as the mean of the mating combination is reduced. The means of the offspring have a direct relation to the grade of the male parent if a comparison is made of varying grades of males when mated to females of the same grade. The same is true if the female grade is varied while her mates' grades are held constant. The rather wide difference between the mean of offspring from the darkest and lightest males indicates the possibility of fewer factors being concerned or, at least, a more clean cut segregation of those involved.

The distribution of color grades for the offspring (table 2) includes all five grades in most cases and bears out the earlier statement concerning a wide range in the amount of black in the primaries and secondaries of different birds.

Bittenbender [9] stated that individuals carrying a dark-red color often have an excess of black in wing. This would indicate that black in wing is associated with the intensity of surface pigmentation. Table 3 data show a negative correlation of -0.013 ± 0.021 to exist between the intensity of surface pigmentation and the degree of black in secondaries. A correlation of such small magnitude is not significant and shows that there is no association between the degree of pigmentation of these two regions.

The amount of black in the wing may be controlled by factors which modify the Columbian pattern found in the Rhode Island Red, and these factors probably have nothing in common with those controlling the intensity of red in the surface.

INHERITANCE OF EYE COLOR

Upon examining the eyes of Rhode Island Red fowls, one will find all variations in color, from a light gray (grade 1) to the reddish-bay (grade 6) iris which is most desired. Differences in distribution as well as kinds of pigment in the eye made the classification of the variations difficult. The grades indicated primarily the amount of red in the iris. The eye color standards [10] show that the reduction in red color begins at the margin of the pupil and progresses toward the outer margin of the iris. Grade 1 occurred so infrequently that no provision was made for this group in the tables. Observations also indicated that there was no change in eye color associated with age. The eye color variations were not associated with any variations in plumage color.

Bond [11] in describing eye color in several breeds states that daw eye (no red) acts as a recessive to a black pigment iris in pigeons and

[9] BITTENBENDER, H. A. See footnote 3.
[10] WARREN, D.C. See footnote 6.
[11] BOND, C. J. ON CERTAIN FACTORS CONCERNED IN THE PRODUCTION OF EYE COLOUR IN BIRDS. Jour. Genetics 9: [69]–81. 1919.

fowls. Further work on the genetics of eye color in poultry is not available.

An examination of table 2 discloses no pronounced sexual dimorphism with regard to eye color. The results here are much less definite than those obtained from the study of black in flight feathers. It is also seen that there is a slight tendency for the mean grade of the offspring to vary with that of the parents. There is, however, considerable lack of consistency in the agreement. Although there is a rather wide range of eye color in the stock considered, the means of eye color for offspring from the most extreme parental combinations do not differ greatly.

The general trend of the results may be taken as evidence of some heritability of eye-color variation in the Rhode Island Red. The evidence is less definite than that found for any of the other color factors studied except that for under color. This may be taken to indicate that some environmental factors are acting to obscure the heritable tendencies. No such environmental factors were identified.

DISCUSSION OF RESULTS

It has not been possible to place the variations in plumage and eye color in the Rhode Island Red on a definite genetic factorial basis. Such results are more or less to be expected when dealing with a quantitative character of this type. The results are further complicated by the necessity of classifying the variations in arbitrary grades.

The method by which the standard color has been acquired in any breed of fowl would tend to bring about a complicated genetic constitution. The poultryman has set up an ideal and accumulated within the breed any hereditary variations which have produced a change in the direction of the ideal. This method would tend to establish in the breed a number of modifying factors. The accumulation of these factors has proceeded over a considerable period of years, and anyone who attempts to unravel the skein of germ plasm should expect to find it a much tangled one.

All the birds considered in this study had a color characteristic of the breed, and the variations considered were those which measured the closeness of approach to the ideal color. It is well known that two major factors are concerned in the production of the Rhode Island Red pattern. These are the sex-linked gold which determines the ground color, and the factor for Columbian pattern which acts in restricting the expression of black to the extremities. It is evident that we have well fixed in this breed other factors darkening the gold color, since the buff varieties, which are much lighter in color, are also known to carry the gold factor. The factors which have been considered are those affecting the intensity of the gold color, and they are not well fixed in the breed. The factors influencing the amount of black in the flight feathers are those modifying the restricting action of the factor for Columbian pattern on black.

The fact that the intensity of color in the various sections of the plumage has been considered separately does not necessarily indicate that color in these regions shows independent inheritance. The back, hackle, breast, and under surface were the sections of the plumage which showed greatest contrast in color. The general re-

sults tend to support the hypothesis that one major factor controls color in all the regions mentioned. The correlation studies showed a fairly high degree of association in the intensity of pigmentation for these sections of the plumage. The summaries of the results in all cases show the males to be darker than the females. Also wherever there was any evidence for dominance or recessiveness the lighter color appeared to be dominant. It is suggested that the difference in intensity of color in the various sections of the plumage may be due to the action of a general pattern factor in the breed.

Although the exact number of factors concerned in color inheritance in this breed could not be determined, there is definite evidence that intensity of color in the back, hackle, and breast, and the amount of black in the flight feathers are inherited. The evidence favoring the inheritance of intensity of color in the under surface of the plumage is much less definite.

In the case of the amount of black in the flight feathers there is a very close agreement in the mean grade of the parents and that of the offspring. The results seem to supply unquestionable evidence that variations in the amount of black in these feathers are inherited but also indicate that several factors are probably concerned in their transmission. Correlation studies show that there is no association between the intensity of pigmentation in the general body color and the amount of black in the wing feathers.

The variations in eye color in this breed do not show definite evidence that they are inherited. The general trend of the results seems to suggest their heritability, but the difference in results when extremes of color are mated is not great.

In all sections of plumage studied and also in eye color, the matings were of such a nature that tests could be made for evidence of sex linkage of the factors concerned. In no case is there evidence that any of the factors involved had their genes carried by the sex chromosome.

SUMMARY

Although there are differences in the shade of red color in four sections of the plumage the general surface, hackle, breast, and under surface of the Single Comb Rhode Island Red fowl, no evidence was found for important independent factors affecting color in these different regions.

Evidence was found for the heritability of the variations in intensity of pigmentation of the general surface, hackle, and breast, but the results support the view that it is a multiple-factor situation. The evidence for heritability of under color was not so definite.

Variations in amount of black in the flight feathers were found to be inherited, but the degree of black in the wings was found to be independent of the intensity of pigmentation of the general surface.

Results on the study of eye color did not seem to indicate that the variations in eye color are inherited.

Sexual dimorphism was observed in plumage-color variations, the males averaging darker than the females in all sections.

None of the characteristics studied showed any evidence that sex-linked factors are concerned.

RECOVERY OF PHOSPHORUS FROM PRAIRIE GRASSES GROWING ON CENTRAL OKLAHOMA SOILS TREATED WITH SUPERPHOSPHATE [1]

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INTRODUCTION

The fact that soils differ considerably in their ability to fix phosphorus from an applied phosphate fertilizer has been known to soil investigators for some time. The efficiency of such fertilizers has been recognized as being low, although actual recovery figures as determined from plant yields and analyses are not very numerous in the literature. Buie [3] estimated that only 10 to 35 percent of the additional phosphoric acid added to the cotton crop was utilized the first year. Russell (*7*, *8*)[4] presents data which indicate a very low recovery from several phosphorus carriers on some English grasslands. Penetration tests by different workers (*2*, *3*, *4*, *6*, *9*, *10*) indicate that most of the phosphorus from an applied phosphate fertilizer never moves a great distance away from the point at which it is applied, although in light soils and in soils receiving manure penetration is greater.

It is the purpose in this paper to present some data on the removal of phosphorus by native prairie grass from virgin soils treated with superphosphate and other fertilizers. Data are also presented on the penetration of phosphorus in one of these soils as measured by the amount present in a more or less easily soluble form in different parts of the profile.

THE SOIL AREAS

Two different soils, one common in the central cross timbers of Oklahoma and the other common in the central prairie section, in which there is slightly less rainfall, were selected for these studies. The soils had apparently never been disturbed from their virgin state, and on each was growing a uniform stand of native prairie grass. *Andropogon scoparius* Michx. was the dominant species, but *A. furcatus* Muhl., *Panicum virgatum* L., *Sorghastrum nutans* (L.) Nash, and *Bouteloua hirsuta* Lag. were common in the area.

The central cross-timber soil selected is a rather dark friable loam at the surface, but changes to a yellowish-brown color at a depth of 3 to 5 inches. The subsoil is a friable clay and is underlain at a depth of 2 to 3 feet with yellowish-brown sandstone. The dark color of the surface is due to the accumulation of organic material from the native vegetation. This soil has not been given a series name as yet, but

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[2] The writer acknowledges his indebtedness to B. F. Kiltz and H. A. Daniel for assisting in some of the collecting and in the analytical work, and to Dr. H. J. Harper for criticisms and suggestions in the preparation of the paper.
[3] Buie, T. S. Private communication.
[4] Reference is made by number (italic) to Literature Cited, p. 916.

is rather common in the east-central portion of the State. The surface soil contains 0.055 percent total nitrogen, 0.019 percent total phosphorus, and 0.0115 percent replaceable potassium. The replaceable potassium was determined by the neutral normal ammonium acetate method. This soil is acid in reaction and is not very productive.

The central prairie soil is classified as Vernon sandy loam, shallow phase. It has a friable clay subsoil and is underlain with sandstone at a depth of about 3 feet. The surface 3 inches is neutral to slightly acid, the 3- to 12-inch horizon is slightly acid, while at about 12 inches and below the soil is slightly basic in reaction. It is considered only fairly productive under the usual climatic conditions common to the area. Both soils are deficient in phosphorus.

METHODS

Two series of plots were laid out on the central cross-timber soil, one early in the spring of 1929, and the other in the spring of 1930. Each plot was one fortieth acre in size (1 rod by 4 rods) and all fertilizer treatments were duplicated, unless otherwise stated. The plots lay side-to-side in each series with one complete set of treatments followed by the duplicate set. The fertilizers were applied broadcast early in April at the beginning of each test and no further applications were made during the tests, which were concluded in the autumn of 1932. The plots on the Vernon soil series were laid out early in the spring of 1930 and were similar in size, treatment, and duplication to those started on the cross-timber area of the same year, except that some duplicated plots of other fertilizer treatments were added and the calcium nitrate treatments were omitted. The plots lay in three tiers with no similar treatments having end-to-end plots. Every sixth plot was used as a check.

The grass was mowed during the latter part of July each year and the field dry weights of hay were recorded. Composite samples of hay from each plot were taken and placed in an electric oven at 105° C. for 48 hours, at which time the samples were weighed and the yields were recalculated to a 10-percent moisture basis. The dried samples were ground to pass through the 0.5-mm sieve of a Wiley laboratory mill and were analyzed for phosphorus. The phosphorus was determined by the official method, magnesium nitrate being used in the preparation of the solution as recommended by the Association of Official Agricultural Chemists (*1*).

The samples of soil used were composite samples made by mixing equal amounts of soil taken from five different locations on each plot. The samples were collected from the various depths by means of soil tubes. The easily soluble phosphorus content was determined by the fifth-normal sulphuric acid method described by Harper (*5*, *p. 4*).

The hydrogen-ion concentration of the soil was determined electrometrically, using the quinhydrone electrode. The dilution was 1 part of soil to 2 parts of water.

RESULTS AND DISCUSSION

A summary of the results, given in tables 1 and 2, indicate as a whole a very small removal of phosphorus by prairie grass from both types of soil. If it is assumed that all the extra phosphorus removed came from the applied superphosphate, the maximum recovery in 4 years was 24.7 percent. The smallest recovery in 3 years was less than 3 percent. These figures represent the total gain in removal of phosphorus over check or nitrogen plots, but apparently this extra phosphorus came almost if not entirely from the applied superphosphate. There was very little difference between the percentage of phosphorus in the hay grown on the plots treated with nitrogen alone and in that grown on the check plots. Furthermore, the percentage of phosphorus in the hay grown on the plots that received phosphate alone and in that grown on the plots that received phosphate in combination with nitrogen was practically the same when the same amount of phosphorus was applied. In general, the use of superphosphate in addition to nitrogen increased the yield of hay. Such was not the case, however, with cyanamid on both soils nor with urea on the Vernon soil, where the yields were not so large as when nitrogen was used alone; yet the percentage of phosphorus was of the magnitude expected for phosphorus treatments.

TABLE 1.—*Removal of phosphorus from central cross-timber soil by prairie grass where different rates and ratios of nitrogen and phosphorus were used*

Treatment per acre (pounds)	Total hay yield for 4 years	Phosphorus in hay	Phosphorus removed	Phosphorus added	Phosphorus recovered [a] in 4 years
	Pounds	*Percent*	*Pounds*	*Pounds*	*Percent*
Checks (average of 5 plots)	8,824	0.0567	5.002	0	0
100 ammonium sulphate+50 superphosphate [b]	8,795	.0603	5.306	4.366	6.96
100 ammonium sulphate+100 superphosphate [b]	9,157	.0607	5.557	8.732	6.36
100 ammonium sulphate+200 superphosphate [b]	9,461	.0736	6.963	17.465	11.23
300 15:15:15:15 [c]	10,951	.0885	9.688	19.648	23.85
300 nitrate of soda 200 superphosphate 100 muriate of potash	11,305	.0702	7.942	17.465	16.83
500 2:12:6 [c]	9,665	.0795	7.686	26.197	10.24
300 superphosphate	10,413	.0854	8.890	26.197	14.84
300 ammonium sulphate+150 superphosphate	11,739	.0756	8.882	13.099	24.70
300 ammonium sulphate+300 superphosphate	13,913	.0820	11.417	26.197	22.03
300 ammonium sulphate+600 superphosphate	14,010	.0929	13.025	52.394	14.08
300 ammonium sulphate	10,042	.0562	5.646	0	0

[a] The term "recovered" is used here and in table 2 to indicate the difference between the amount of phosphorous removed from the nitrogen and phosphorus plot and from the nitrogen plot, where data are available. Where there is no similar nitrogen treatment the check-plot data are used. This difference is compared with the quantity of phosphorus added to the soil in the fertilizer which gives the percentage indicated in column 6. It is recognized that such a comparison may be subject to errors because some plants have more extensive root systems than others and hence greater soil volume in which to secure nutrients as well as perhaps an increased ability to secure plant foods from crude material; yet, by making use of the nitrogen plots in determining the differences in uptake of phosphorus, these are probably taken care of as well as experiments of this type can afford. It is certain that the recovery from the applied superphosphate does not exceed the percentage given.

[b] The superphosphate contained 20 percent P_2O_5.

[c] The first number refers to the percentage of nitrogen, the second to percentage of P_2O_5, and the third to the percentage of K_2O in the fertilizer.

TABLE 2.—*The 3-year removal of phosphorus from central cross-timber soil by prairie grass and from Vernon sandy loam*

Treatment [b]	Total yield of hay per acre	Phosphorus in hay	Phosphorus removed	Phosphorus added	Phosphorus recovered in 3 years
CROSS-TIMBER SOIL [a]					
	Pounds	*Percent*	*Pounds*	*Pounds*	*Percent*
Check (average of 4 plots)	5,531	0.0428	2.365	0	0
Calnitro	7,334	.0449	3.293	0	0
Ammonium sulphate	7,117	.0442	3.144	0	0
Nitrate of soda	7,068	.0438	3.097	0	0
Calurea	7,040	.0399	2.809	0	0
Urea	6,667	.0419	2.794	0	0
Cyanamid	7,777	.0453	3.525	0	0
Calcium nitrate	7,597	.0404	3.070	0	0
Calnitro+superphosphate	8,216	.0545	4.481	17.465	6.80
Ammonium sulphate+superphosphate	8,982	.0550	4.943	17.465	10.30
Nitrate of soda+superphosphate	8,859	.0540	4.788	17.465	9.68
Calurea+superphosphate	7,867	.0545	4.289	17.465	8.47
Urea+superphosphate	7,286	.0565	4.114	17.465	7.56
Cyanamid+superphosphate	7,061	.0568	4.014	17.465	2.88
Calcium nitrate+superphosphate	8,774	.0504	4.420	17.465	7.73
VERNON SANDY LOAM [c]					
Check (average of 8 plots)	4,635	0.0537	2.590	0	0
Calnitro	4,063	.0461	1.875	0	0
Ammonium sulphate	4,624	.0488	2.257	0	0
Nitrate of soda	5,418	.0485	2.630	0	0
Calurea	5,326	.0450	2.397	0	0
Urea	6,268	.0530	3.324	0	0
Cyanamid	5,768	.0491	2.835	0	0
Calnitro+superphosphate	5,817	.0664	3.865	17.465	11.39
Ammonium sulphate+superphosphate	5,923	.0617	3.658	17.465	8.02
Nitrate of soda+superphosphate	5,761	.0555	3.197	17.465	3.25
Calurea+superphosphate	6,038	.0664	4.012	17.465	9.25
Urea+superphosphate	6,089	.0677	4.125	17.465	4.69
Cyanamid+superphosphate	5,499	.0674	3.709	17.465	5.00
Superphosphate	5,052	.0635	3.211	17.465	3.56
15:30:15, 267 pounds per acre	6,658	.0833	5.550	34.929	9.09
0:46:0, 364 pounds per acre	5,805	.1019	5.920	83.027	4.01
11:46:0, 364 pounds per acre	7,406	.0958	7.099	83.027	5.69
10:30:10, 400 pounds per acre	7,404	.0822	6.086	52.394	7.08
12:24:12, 333 pounds per acre	6,815	.0819	5.586	34.929	9.19

[a] The average phosphorus content of the hay for the treatments shown are: Checks 0.0428 percent; all nitrogen treatments 0.0429 percent, and all nitrogen and phosphorus combinations 0.0545 percent.

[b] All nitrogen fertilizers were applied at the rate of 40 pounds of nitrogen per acre. The superphosphate contained 20 percent P_2O_5 and was applied at the rate of 200 pounds per acre.

[c] The average phosphorous content of the hay for the treatments shown are: Checks, 0.0537 percent; all nitrogen treatments, 0.0484 percent; superphosphate alone, 0.0635 percent; and all nitrogen and superphosphate combinations, 0.0642 percent.

When nitrogen was applied with superphosphate there was an increase in yield as compared with that when superphosphate alone was used; nevertheless, the percentage of phosphorus in the hay remained practically the same. In the surface 12 inches of untreated cross-timber virgin soil (table 3), the easily soluble phosphorus, i.e., the phosphorus soluble in 0.2 N H_2SO_4, was less than that in the treated soil when both nitrogen and phosphorus were applied, but below this portion of the profile there was little difference. The surface few inches of Vernon soil 2 years after the application of superphosphate showed (table 4) more soluble phosphorus than did the untreated soil. For most of the treatments the increase was confined mostly to the surface 3 inches. It thus appears that as nitrogen is furnished to the plant to increase yield, the plant makes use of the small extra amount of more easily soluble phosphorus left in the surface few inches of soil by the applied superphosphate, and so the percentage of phosphorus recovered from the fertilizer is increased.

The increase in yield is probably the result of many factors. It is suggested that a larger root system may contribute considerably in this respect. If such is the case it is probable, though not proved, that the root system becomes more extensive in the surface soil volume instead of extending down into the subsoil of low fertility. It does not appear that the nitrogen influenced phosphorus absorption except as it might have increased the yield of hay, since the percentage of phosphorus in the hay for any of the nitrogen treatments was of the same order of magnitude as that in the hay produced on unfertilized soil.

No significance is to be attached to the difference in the amount of phosphorus removed in the case of the various nitrogen-phosphorus combinations. The yields of the plots and duplicates were as uniform as could be expected, but with so small a removal as was secured in any one case slight variations in yield would account for the differences obtained. This is emphasized by the fact that no one nitrogen carrier proved best on the two types of soil studied.

TABLE 3.—*The pH and soluble phosphorus at various depths 2 years and 3 years after application on the central cross-timber soil*

2 YEARS

Treatment per acre	0-3 inches		3-6 inches		6-12 inches		12-24 inches		24-36 inches	
	pH	Soluble phosphorus	pH	Soluble phosphorus	pH	Soluble phosphorus	pH	Soluble phosphorus	pH	Soluble phosphorus
		P.p.m.		*P.p.m.*		*P.p.m.*		*P.p.m.*		*P.p.m.*
Check	6.44	3.0	6.48	2.5	6.44	1.0	6.39	1.4	6.58	1.2
Calnitro+superphosphate	6.63	11.0	6.63	1.0	6.59	1.0	6.34	1.0	6.35	1.6
Ammonium sulphate+superphosphate	6.42	5.0	6.35	1.0	6.32	2.0	6.20	2.0	6.86	1.4
Nitrate of soda+superphosphate	6.58	4.0	6.58	1.5	6.34	3.0	5.83	1.2	6.42	1.0
Calurea+superphosphate	6.27	3.5	5.59	1.5	5.61	3.0	5.78	1.5	5.78	1.2
Urea+superphosphate	6.44	6.0	6.04	1.0	5.96	3.0	5.88	1.0	5.86	1.5
Cyanamid+superphosphate	6.35	3.5	5.99	1.5	5.99	.5	6.09	Trace	6.04	1.5
Calcium nitrate+superphosphate	6.04	3.0	6.13	1.5	5.80	3.0	5.82	1.7	5.96	2.0
Check	6.30	3.0	6.40	1.5	6.00	Trace	5.80	1.2	6.01	1.2

3 YEARS [a]

Check	6.23	3.0	6.04	2.5	5.54	1.0	5.24	0.8	5.47	1.5
100 pounds ammonium sulphate, 50 pounds superphosphate	6.34	3.0	6.28	3.0	5.76	2.0	5.33	1.5	5.57	2.0
100 pounds ammonium sulphate, 100 pounds superphosphate	6.18	3.0	6.30	1.0	5.75	1.5	5.78	1.5	5.68	1.0
100 pounds ammonium sulphate, 200 pounds superphosphate	6.35	4.0	5.71	2.0	5.75	1.0	5.61	1.2	5.86	1.0
300 pounds 15:15:15 fertilizer	6.30	3.0	6.56	2.5	5.86	1.5	5.35	1.5	5.92	1.0
Check	6.68	3.0	6.70	2.0	6.01	1.0	6.49	1.2	5.57	1.0
300 pounds nitrate of soda, 200 pounds superphosphate, 100 pounds muriate of potash	6.30	5.0	6.09	2.0	5.76	1.5	5.71	.8	5.70	1.2
300 pounds ammonium sulphate, 150 pounds superphosphate	6.11	4.0	5.71	2.5	5.54	2.0	5.83	1.5	5.71	1.0
300 pounds ammonium sulphate, 300 pounds superphosphate	6.30	3.5	6.11	1.0	5.75	1.5	5.33	.8	5.40	1.0
300 pounds ammonium sulphate, 600 pounds superphosphate	5.96	9.0	5.94	1.5	5.37	1.5	5.12	1.0	5.23	1.7
Check	6.09	3.5	5.57	2.0	5.61	1.5	5.54	1.0	[b]	[b]
300 pounds superphosphate	6.11	3.0	5.88	2.0	5.96	[c]	6.06	1.2	[b]	[b]
500 pounds 2:12:6 fertilizer	6.32	4.0	6.04	1.7	5.86	1.5	6.37	1.5	[b]	[b]

[a] The composite sample of the soil at the beginning of the experiment had a pH of 6.78 and contained 3 parts per million of soluble phosphorus in the surface 3 inches.
[b] No samples were taken on account of sandstone.
[c] Trace.

TABLE 4.—*The soluble phosphorus content of the surface 6 inches of Vernon soil 2 years after applying the fertilizers*

Treatment	Phosphorus soluble in 0.2 N H_2SO_4	
	0–3 inches	3–6 inches
	P.p.m.	*P.p.m.*
Checks (average of 8 plots)	8	6
Calnitro+superphosphate	8	8.5
Ammonium sulphate+superphosphate	13	5
Nitrate of soda+superphosphate	12	10.5
Calurea+superphosphate	11.5	6.5
Urea+superphosphate	11	11
Cyanamid+superphosphate	10.5	6.5
Superphosphate	11.5	6.5

SUMMARY AND CONCLUSIONS

This paper reports results secured in field trials to determine the quantities of phosphorus removed from two common pasture and grassland soils in central Oklahoma by native prairie grasses consisting mainly of *Andropogon scoparius*. The natural phosphorus content of these grasses is low.

The use of superphosphate on these soils increased the yield of hay and raised the phosphorus content of the hay produced. When nitrogen as well as phosphorus was applied in the fertilizer the percentage of phosphorus in the hay was of the same order of magnitude as when superphospate only was applied.

When nitrogen and superphosphate were applied the yield of hay in all cases was greater than when superphosphate alone was used, but it was not always greater than when nitrogen was used alone. The percentage of phosphorus in the hay produced on the plots that received nitrogen alone was of the same order of magnitude as that in the hay produced on unfertilized soil.

Regardless of whether the soil furnished any extra phosphorus for the superphosphate, nitrogen treated plots beyond that furnished on the nitrogen plots, the recovery of phosphorus from the applied superphosphate was very small.

If the amount of phosphorus soluble in 0.2 N H_2SO_4 can be taken as a guide, the phosphorus applied did not penetrate to any great distance in the cross-timber soil, being confined largely to the surface 3 inches; certainly it did not penetrate to a depth exceeding 12 inches.

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